Molecular genetic analyses in acquired epilepsies

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Erklärung

Diese Dissertation wurde im Sinne der Promotionsordnung vom 17.06.2011 von Prof. Dr. Albert J. Becker betreut.

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1 Summary

Focal epilepsies represent multifactorial disorders. Hence, pathogenetic factors, episodically shifting the brain over a virtual threshold to the emergence of seizures, are *individually* neither necessary nor sufficient. In recent years, several SNPs located in potential promoter regions of related genes have been detected in patients suffering from episodic CNS disorders. Transiently altered expression of corresponding genes therefore constitutes a potential pathogenetic aspect for the manifestation of episodic symptoms. The availability of biopsy tissue from epilepsy surgery of pharmacoresistant TLE patients provides a unique prerequisite in order to study the potential impact of gene promoter variants on transcription as well as the correlation of gene expression involved in neurotransmission and immune responses corresponding to stratification of patients according to clinical parameters.

The focus of this study was to gain further insights on the potential impact of SNPs located in transcriptional regulatory regions to modulate the expression of respective genes coding for neurotransmitter receptors including serotonin receptors and genes related to inhibition and neurotransmission, i.e. genes involved in γ-aminobutyric acid (GABA)-ergic homeostasis, on the basis of human surgical hippocampal brain tissue. By using real-time RT-PCR we found differential mRNA expression levels of relevant genes corresponding to the presence of respective SNP genotypes. To unravel the mechanisms of altered promoter control via regulatory SNP influence or aberrant transcription factor effects, we performed comprehensive bioinformatic analysis in order to identify binding sites for transcription factors and their potential modification by promoter SNPs. We observed that respective promoter SNPs affect transcription factor binding. Additionally, we showed an allele-dependent regulation of gene expression after exposure to relevant transcription factors using luciferase reporter asays.

Furthermore, given the potential impact of seizure frequency on gene expression, we analyzed the correlation of gene expression levels in surgical hippocampi from TLE

patients with clinical or functional parameters. We found a significant correlation of expression of distinct mediator genes of inflammation to seizure frequency in human surgical brain tissue of pharmacoresistant TLE patients.

Our data indicate novel insights in the relevance of dynamic expression of genes related to neurotransmission and inflammation based on human brain tissue of TLE patients not responding to antiepileptic drugs.

2 Introduction

2.1 Epilepsy

Epilepsies belong to the most common neurological disorders in humans and are characterized by spontaneous appearance of two or more unprovoked seizures due to recurrent abnormal excessive or synchronous neuronal activity in the brain (Fisher et al., 2005). About 10% of the population are exposed to one seizure during their lifetime (Hauser et al., 1990). Epileptic seizures generate either focal or generalized paroxysomal changes, which are induced by excessive, abnormal or synchronous neuronal activity in the brain (Hauser and Kurland, 1975; Dichter, 1994; Sanabria et al., 2001; Fisher et al., 2005; Fritschy, 2008; Banerjee et al., 2009). In generalized epilepsy, seizures are not restricted to a particular brain region. In focal epilepsy, the abnormal neuronal activity onset is restricted to one defined brain region, the so called *epileptic focus*.

A proportion of 0.5 to 1% of the population suffer from chronic epilepsy (Hauser et al., 1996; Elger, 2002). Epilepsies can be divided in *symptomatic* and *idiopathic* syndromes. The genetically defined *idiopathic* epilepsies manifest without structural or other predisposing cause. Both focal and generalized forms of epilepsy can be caused by genetic defects, e.g. in genes coding for voltage gated sodium, potassium channels or $GABA_A$ receptor chloride channels, called channelopathies (Heron et al., 2007). The most common form constitutes the *idiopathic generalized epilepsy* (IGE) comprising *juvenile myoclonic epilepsy* (JME) and *childhood absence epilepsy* (CAE). *Juvenile myoclonic epilepsy* (JME) is usually featured by first seizure onset between the ages of 12 and 18 and seizure episodes occurring after a sleep period. JME is characterized by myoclonic, generalized tonic-clonic seizures and, infrequently, absence seizures (Genton and Gelisse, 2001). *Childhood absence epilepsy* (CAE) constitutes 10 - 17% of all cases of childhood-onset epilepsy and typically begins at 4 - 10 years with a peak at age

between 5 - 7 years (Berg et al., 2000). These patients have frequent absence seizures. *Symptomatic* epilepsies are caused by acquired or native structural or metabolic defects of the brain, e.g. perinatal or postnatal trauma, infections of the central nervous system (CNS) or cerebrovascular lesions. These forms of epilepsies mainly have a focal origin due to e.g. tumors, stroke or hippocampal sclerosis. The latter is major pathology in temporal lobe epilepsy (TLE).

2.1.1 Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is one specific form of epilepsy characterized by focal seizures, which can secondarily generalize. TLE represents the most common form of acquired epilepsy in humans and affects approximately 70% of all epilepsy patients (Engel, 1996a; Sirven, 2002). In TLE, the hippocampus often shows the pathology of Ammon's horn (or hippocampal) sclerosis (AHS). About 35% of the TLE patients have focal lesions such as benign glial and glioneuronal tumors as well as cortical malformations (Blümcke et al., 1996; Thom, 2004). 5 - 20% of epilepsy patients manifest a structural or focal lesion together with AHS, which is indicated as a 'dual pathology' of epilepsy (Wieser, 2004). With regard to TLE etiology, febrile seizures in early childhood often correlate to the development of TLE. However, also brain insults, stroke, infections of the CNS as well as malformations in the cortical development or brain tumors can act as initial event of epileptogenesis (Brooks-Kayal et al., 2009; Pitkanen et al., 2009; Rakhade and Jensen, 2009).

2.1.1.1 Ammon's horn sclerosis

The hippocampal formation is localized in the temporal lobe and belongs to the limbic system. The hippocampus is responsible for consolidation of short-term and long-term memory information, emotions and spatial navigation (Scoville and Milner, 1957; Nunn et al., 1999). The hippocampal formation consists of the *dentate gyrus*, the *subiculum* and the *cornu Ammonis* (Ammon's horn), which itself comprises the hippocampal

regions CA1 to CA4. In the case of TLE patients, the pattern of AHS as most common neuropathological finding is diagnosed in about 60% of epilepsy-surgical resections (Blümcke et al., 2002; Majores et al., 2007). The segmental neuronal cell loss in the hippocampal formation is defined according to its distribution pattern in four different types relevant to the Blümcke classification (Blümcke et al., 2007). The classical pattern of AHS is characterized by a severe nerve cell loss in CA1 and a moderate loss in CA2, CA3 and CA4 (type 1A according to Blümcke). A massive neuronal cell loss in all hippocampal regions is classified as type 1B according to Blümcke. Type 2 shows a striking neuronal cell loss in CA1 (CA1 sclerosis), whereas in the so called *endfolium sclerosis* (type 3 according to Blümcke) neuronal cells are mostly preserved in CA1 and a massive cell loss is detectable in CA2, CA3 and CA4. The degree of severity of the hippocampal sclerosis is significantly influenced by several factors such as duration of epilepsy, the age of patients at seizure onset and the occurrence of febrile seizures in early childhood (Davies et al., 1996; Blümcke et al., 1999; Janszky et al., 2005; von Lehe et al., 2006).

2.1.2 Pharmacoresistant epilepsies and epilepsy surgery

In many patients, seizures in epilepsy are well treatable with existing antiepileptic drugs (AEDs). However, in a significant number of patients no response to any of these several diverse acting drugs occurs. These patients have to be designated as *pharmacoresistant*, if the treatment with two ore more AEDs does not lead to seizure control. In particular, TLE patients are known to frequently generate pharmacoresistance (30%) (Engel, 1996b; Regesta and Tanganelli, 1999).

To date, two main concepts are established for explaining the molecular cause of pharmacoresistance: first, aberrant function and upregulation of multidrug transporters at the blood-brain barrier (BBB) leads to a decreased and therefore insufficient concentration of AEDs in the brain parenchyma ('transporter hypothesis'; Kwan and Brodie 2005). Secondary, the so called 'target hypothesis' is based on potential alterations of

AED targets, such as neurotransmitter receptors or voltage-gated ion channels (Heinemann et al., 2006; Remy and Beck, 2006).

In pharmacoresistant patients, the required alternative therapy is given by the opportunity of surgical resection of the epileptic focus. Electroencephalogram (EEG) video monitoring, neuropsychological evaluation and invasive electrophysiological methods are used for mapping of the epileptic focus. The removal of the epileptic focus provides remarkable prospects for the TLE patients. In 70% of the cases, patients become seizure free, for further 20% of the patients epilepsy surgery results in considerable seizure reduction ($< 90\%$) and only 10% of the patients do not benefit from epilepsy surgery (Engel et al., 1993).

Epilepsy surgery provides the opportunity to obtain fresh human epileptic brain tissue for molecular experiments.

2.2 Inflammation

Naturally, inflammation is part of the immune system defense against injury and disease. Chronic brain inflammation includes activation of microglia, astrocytes, endothelial cells of the BBB and peripheral immune cells as well as accompanied production of inflammatory mediators such as in the epileptic syndrome of Rasmussen's encephalitis (Rasmussen et al., 1958). Mediators are produced from tissue-resident or blood-circulating immunocompetent cells during a dynamic process and are involved in activation of the innate and adaptive immunity (Vezzani et al., 2011).

Increasing evidence indicates that inflammation plays a prominent role in the pathophysiology of a number of human epilepsies and convulsive disorders (Wirrell et al., 2005; Bauer et al., 2009). Furthermore, numerous studies suggest that inflammatory processes in the CNS are either caused by or contribute to epileptogenesis (Vezzani et al., 2002; Vezzani and Granata, 2005). This is emphasized by the detection of inflammatory mediators, for example interleukins, interferons, chemokines or tumor

necrosis factors, in surgical brain tissue from patients with refractory epilepsies (Vezzani and Granata, 2005; Choi et al., 2009). Experimental evidence indicates that seizure activity can lead to initiation of brain inflammation (Vezzani and Granata, 2005; Riazi et al., 2010). Additionally, as previously reported, a prolonged proinflammatory state in the CNS may contribute to seizure predisposition and occurrence. This in turn is associated with changes in neuronal excitability and enhanced seizure-induced neuropathology (Ravizza et al., 2008; Vezzani and Granata, 2005). Furthermore, this aspect is highlighted by previously reported alterations in seizure susceptibility in genetically modified mice with impaired inflammatory signaling (Sarro et al., 2004; Balosso et al., 2005).

2.3 Promoter characteristics and detection

The typical structure of an eukaryotic gene promoter consists of regulatory sequences (Figure 1) such as core elements and several enhancer or silencer elements distributed at various distances from the transcription start site (TSS) of the gene (Wasserman and Sandelin, 2004; Halfon, 2006). The *core* promoter is often located approximately 100bp upstream of the first exon and comprises the initiator element (INR) and an AT-rich site located 25 - 30bp upstream of the TSS, known as TATA-box. The *proximal* promoter region harbors binding sites for special functional proteins that control gene transcription rates. Especially in eukaryotes, regulatory elements such as enhancers, repressor elements and silencers which influence the transcription independent of their orientation, are often located up to 85 kb from the TSS (*distal* promoter) (Blackwood and Kadonaga, 1998; Lin et al., 2007; Kuttippurathu et al., 2011).

Repressors interact with the Deoxyribonucleic acid (DNA) sequence in order to affect the transcription of a corresponding gene. Repressors can act in the classical fashion in an orientation- and position-dependent manner (Ogbourne and Antalis, 1998) or by interfering with the binding of an activator (Hanna-Rose and Hansen, 1996). Proximal

as well as distal regulatory regions are often arranged in clusters, so called cis-regulatory modules, which cover similar signals (Halfon, 2006).

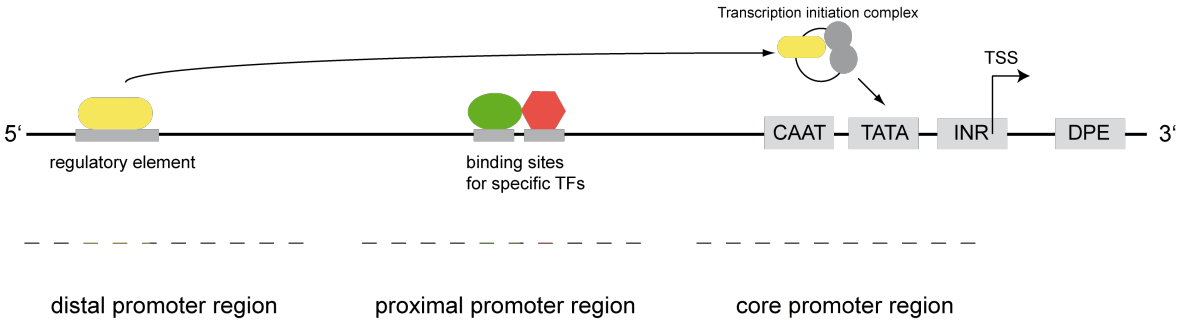

Figure 1: Characteristics of the eukaryotic promoter region. The general promoter in eukaryotes consists of the core, the proximal and the distal promoter region in upstream order relative to the transcription start site (TSS). These regions are characterized by distinct sequence patterns. The core promoter contains several closely located TSSs with different rates of initiation grouped as initiator element (INR), an AT-rich site called TATA box sufficient for directing transcription initiation by a basal transcription complex. General transcription factors are known to bind to the CAAT box. Binding sites for specific transcription factors are located in the proximal promoter region to control gene transcription rates. Up to 85kb upstream of the TSS in the distal promoter region, regulatory elements can influence the gene transcription by e.g. interfering with the transcription initiation complex. In some cases downstream promoter elements (DPEs) are positioned downstream of the TSS (modified from Wasserman and Sandelin 2004).

Known gene promoter regions are generally available in various databases. The Eukaryotic Promoter Database (EPD) comprises annotated non-redundant experimentally identified eukaryotic promoters, whereas the Mammalian Promoter Database (MPromDb) harbors annotated promoters identified from Chromatin Immunoprecipitation Sequencing (ChIP-Seq) experiment results (Périer et al., 2000; Sun et al., 2006). Direct detection of promoters is also possible from genomic sequences. Multiple methods and algorithms have been developed in the past few years to identify promoter regions. To localize the TSS, as one main characteristic of the gene promoter, the software tool Eponine uses a neuronal network model based on position weight matrices (PWMs) for the collection of positioned constraints (Down and Hubbard, 2002). Such positional characteristics comprise a GC-rich region downstream of the potential TSS, TATAAA motifs corresponding to the widely reported TATA box and additional two GC-rich motifs flanking the identified TATA box.

In about 70% of human promoters, long stretches of GC-rich regions, so called CpG is-

lands, are present and therefore denote a often relevant feature of the 5'-region of mammalian genes (Antequera, 2003). EMBOSS Cpgplot from the European Bioinformatics Institute (EBI) at the European Molecular Biology Laboratory (EMBL) presents an efficient tool to find such CpG islands associated with promoters (Rice et al., 2000).
As previously described, eukaryotic promoter regions have an abundance of TATA- as well as CAAT-motifs. Therefore, combining both TATA and CAAT position weight matrices to scan genomic upstream regions, constitute an intriguing option for the discovery of promoter characteristics.
In summary, the combination of the results of multiple distinct promoter detection methods may provide a useful approach in order to designate a potential promoter region for a gene of interest.

2.3.1 Transcription factors and their binding sites

Gene transcription is mainly concerted by a large number of regulatory proteins. Generally, transcription factors (TFs) are classified according to their mechanistic, functional or structural role. Ubiquitously expressed *general* transcription factors including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH represent a mechanistic class of TFs. General TFs play a role in the formation of the preinitiation complex. As second mechanistic class, *specific* transcription factors bind upstream of the TSS to enhance or repress gene transcription (Conaway and Conaway, 1997; Torchia et al., 1998; Maldonado et al., 1999; Näär et al., 2001).
Based on the functional role, so called *constitutive active nuclear* factors with a transcriptional activating function are present in the cell nucleus of all cells at all time. This group comprises SP1, NF1, CCAAT and general transcription factors (Brivanlou and Darnell, 2002). *Conditionally active* TFs depend on an external activation signal. One subgroup is *cell-type specific*, i.e. it requires extracellular signals to be first generated and then enter the nucleus without further regulation by posttranslational signals. So called *signal-dependent* TFs are developmentally limited or present in all cells in

an inactive form until the activation by an intra- or extracellular signal (Brivanlou and Darnell, 2002). Dependent on sequence similarity of their respective DNA-binding motifs, TFs are also grouped in superclasses of basic domains, zinc-coordinating DNA-binding domains, helix-turn-helix domains and beta-scaffold factors with minor groove contacts (Stegmaier et al., 2004).

The function of TFs is reflected by the classical structure consisting of a DNA binding domain responsible for the binding to the DNA sequence and a transcriptional activation domain. The activation domain mediates stimulatory or inhibitory effects on gene transcription by interacting either in a direct fashion with specific components of the basal transcriptional complex or indirectly with co-activators, which then interact with the basal transcriptional complex (Latchman, 1997). Besides transcriptional activation by activating TFs, repressing TFs may act as competitive binding proteins as well as interaction partners of the activating TF to prevent the activity of its activation domain. Likewise, in order to inhibit the activating TF from binding to the target DNA sequence, the repressing TF operate as direct DNA binding partner or by formation of a non-DNA binding complex with the activating TF (Latchman, 1997).

In general, TFs interact with short (typically a length of 6 - 20 bases) *cis*-acting sequence stretches called transcription factor binding sites (TFBSs). It is known that TFs interact in a sequence specific manner, due to the fact that not all nucleotides of the TFBS actually form a bond with the TF and the degree of interaction differs among each other. Furthermore, a single TF is not restricted to one binding motif but has the ability to identify a subset of binding motifs featured by minimal sequence differences. Considering the highly diverse and variable binding motifs for each TF, their detection and characterization represents a challenge.

2.3.2 Prediction of transcription factor binding sites

The detection of TFBSs in the promoter region of a gene constitutes an initial step in order to unravel regulatory mechanisms of gene regulation. Taking into account that

binding motifs for one TF are often similar to each other but not necessarily identical, the motif finding problem may be defined as:

"Given a set of sequences (promoter regions), detect overrepresented motifs that are good candidates for being transcription factor binding sites" (Sinha and Tompa, 2003).

Most motif finding algorithms based on position weight matrices (PWMs), synonymous with position specific scoring matrices (PSSMs), imitate position specific variations such as quantitative preferences between the nucleotides at each position. Therefore, multiple experimentally validated binding sites for one TF are aligned and a position frequency matrix (PFM) is established that contains the frequency of observed nucleotides at each position of the alignment. First, normalized values of the PFM are converted to a log-scale using the formula (1) to finally provide a quantitative description of the already known binding sites for the TF (Stormo, 2000).

$$PWMconversion: \qquad W_{b,i} = log_2 \frac{p(b,i)}{p(b)} \tag{1}$$

where $p(b)$ = background probability of base b; $p(b,i)$ = corrected probability of base b in position i; $W_{b,i}$ = PWM value of base b in position i.

The resulting PWM assumes independence between the positions in the pattern (leftmost in Figure 2). By adding the relevant nucleotide PSSM values at each position, a quantitative score for a potential sequence motif can be generated. This score is proportional to the binding energy of the TF to the DNA (Berg and von Hippel, 1987; Stormo, 2000). A graphical method to determine the consensus sequence as well as the relative frequency of the bases and the information content at every position in a binding site is given by a sequence logo (rightmost in Figure 2; Schneider and Stephens 1990). The sequence logo in Figure 2 showed an instantaneous visual overview of motif characteristics taking the example of the TF Early growth response 3 (Egr-3).

	A	C	G	T
01	17.1	7.3	9.8	65.9
02	1.9	0.0	98.1	0.0
03	0.0	100.0	0.0	0.0
04	0.0	0.0	100.0	0.0
05	0.0	0.0	0.0	100.0
06	32.1	0.0	67.9	0.0
07	0.0	0.0	100.0	0.0
08	0.0	0.0	100.0	0.0
09	5.7	88.7	0.0	5.7
10	0.0	0.0	100.0	0.0
11	2.0	0.0	34.0	64.0

Figure 2: Representative position specific scoring matrix (PSSM) of Egr-3 with corresponding sequence logo. On the left side, the PSSM is illustrated by a matrix with one row for each base and one column for each position in the pattern. On the right side, the sequence logo is composed of a stack for each position in the sequence. The sequence conservation at each position is indicated by the overall height of the stack, while the depth of each single symbol within the stack is made proportional to the frequency of each nucleic acid at that position.

One traditional public data source for PSSMs is named TRANScription FACtor database (TRANSFAC) sponsored by BIOBASE. This database integrates data of eukaryotic transcription factors, their experimentally-proven binding sites as well as consensus binding sequences (Wingender et al., 1996; Wingender, 2008).

2.4 Single nucleotide polymorphism

The comparison of two randomly selected human genomes reveals up to 99.9% sequence identity. The remaining 0.1% consist of sequence variations (Shastry, 2002). These sequence variations comprise besides tandem repeats, such as mini- and microsatellites, large and small segmental deletions, insertions or duplications, so called single nucleotide polymorphisms (SNPs) (Chorley et al., 2008). SNPs are single base substitutions in genomic DNA among different individuals in some populations. SNPs constitute about 90% of all known sequence variations and are depicted to be highly abundant, stable and distributed throughout the genome by occurring at a frequency of approximately 1 in 1000 base pairs (Brookes, 1999). The distribution in the entire

genome ranges over coding regions of genes, non-coding regions and intergenic regions. *Synonymous* SNPs do not alter the encoded amino acid sequence and do not undergo natural selection (Kimura, 1983). Otherwise, *non-synonymous* SNPs change the protein sequence and may be subjects to natural selection.

SNPs are not exclusively present in coding regions but also in regulatory regions such as promoters, enhancers and silencers. They affect gene splicing, messenger RNA (mRNA) degradation, transcription factor binding or promoter activity (Lohrer and Tangen, 2000). Such SNPs are often designated as regulatory SNPs (rSNPs) (Knight, 2005; Wang et al., 2005). Generally, a rSNP occurring in a potential binding site for a TF does neither influence the binding affinity nor alter the corresponding gene expression. However, in some cases the SNP leads to an increase or decrease of the interaction potency of TF and DNA sequence and therefore to allele-specific gene expression. In contrast, the complete elimination of the natural binding site or the generation of a recent binding site due to the occurrence of a SNP appears quite rarely (Figure 3).

Considering the up-to-date huge amount of about 62676337 annotated human SNPs (dbSNP build 138 date April 25, 2013) in the dSNP database of the National Center for Biotechnology Information (NCBI), the importance of SNPs for genetic analyses becomes more and more explicit (Sherry et al., 2001).

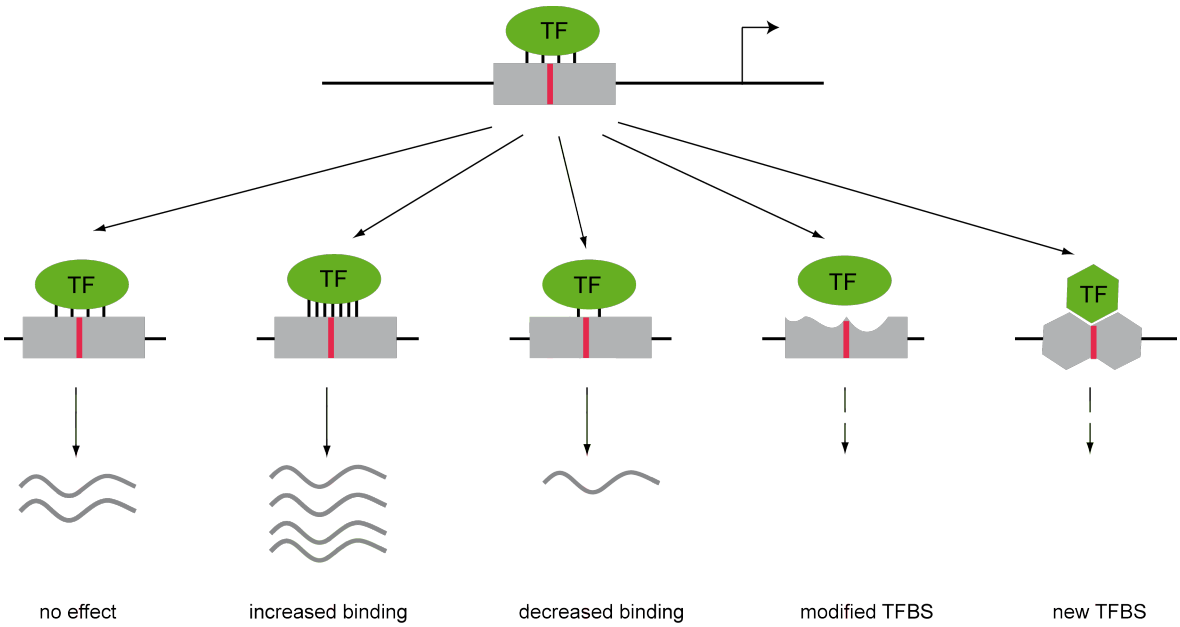

Figure 3: Alternative influences of SNP on TFBS. The rSNP (red bar) is located in a TFBS (grey) and potentially alters the binding of a TF (green) to the TFBS and further gene expression. From left to right: SNP has neither effect on the binding affinity nor alter the corresponding gene expression. Sometimes SNPs increase or rather decrease the binding to TFBS and therefore leads to allele-specific gene expression. In rare cases, the natural binding site of TF is destroyed or a recent binding site is generated (modified from Chorley et al. 2008).

2.5 Integrated investigation of expression analysis and genome-wide association studies

The transcript level of a gene can be influenced by DNA sequence variations such as SNPs, structural variations of large DNA stretches (copy number variants, CNVs), insertions and deletions or short tandem repeats and by epigenetic modifications such as methylation of CpG-residues or post-translational modifications of histones (Eckhardt et al., 2006; Shimada et al., 2009). As mentioned before, SNPs may functionally influence multifactorial diseases, such as epilepsy, by directly altering the abundance of a gene transcript. As a consequence, transcript alterations may be considered as a quantitative trait. Regulatory regions that control levels of gene expression are mapped as expression quantitative trait loci (eQTL) (Schadt et al., 2003; Morley et al., 2004). *Cis*-acting eQTLs mapped proximal to the gene and cover SNPs within 100 kb up- and

downstream of their gene-of-origin. Conversely, eQTLs mapped at long distances from their gene-of-origin are more numerous and referred to as *trans*-eQTLs (Michaelson et al., 2009).

Analysis of eQTLs provides considerable potential for examination of transcriptional regulation control even at a genome-wide scale. Combination of gene expression studies, e.g. in human hippocampal tissue, with genome-wide genotyping of SNPs, represents a possible basis of association studies of genetic markers with individual gene expression. Previous gene expression studies using human hippocampal tissue, indicate biological networks to be significant in gene expression changes such as GABA-ergic transmission, signal transduction and ion transport, glial activation and immune response including inflammation (Aronica and Gorter, 2007).

An additional advantage of genome-wide assocation (GWA) studies is given by reconstruction of gene networks to identify key regulators of complex disease traits for further biological validation (Zhu et al., 2004). Such network analyses including gene expression and genetic data are known to clarify mechanisms underlying multifactorial diseases, e.g. epilepsy (Chen et al., 2008; Emilsson et al., 2008).

2.6 Aims of this work

Amongst other complex disorders of the CNS such as depression and migraine, epilepsies are characterized by episodically aberrant neuronal activity. Mechanisms related to excitability and neurotransmission play a central role. Intriguingly, several susceptibility variants have been detected in the promoter regions of related genes in patients suffering from such neurological disorders (Gratacos et al., 2009; EPICURE Consortium et al., 2012; Cordoba et al., 2012; Duong et al., 2012). However, functional consequences of these variants often remained unresolved.

The aim of this work is to investigate the potential impact of SNPs located in transcriptional regulatory regions on the expression of respective genes coding for neurotrans-

mitter receptors including serotonin receptors as well as genes related to inhibition and neurotransmission, i.e. genes involved in γ-aminobutyric acid (GABA)-ergic homeostasis on the basis of human surgical hippocampal brain tissue.

The first objective is targeted by a candidate-based approach using previously reported associations between promoter SNPs and changes in gene expression. Do promoter variants qualitatively and quantitatively influence corresponding gene expression and as a consequence, aspects of disease susceptibility and generation? We therefore will perform comprehensive functional studies based on our unique access to fresh frozen human surgical hippocampal brain tissue of pharmacoresistant TLE patients. First, we will examine potential allele-specific mRNA expression changes of relevant genes using the real-time RT-PCR approach. We will pay special attention to mechanisms of altered promoter control via regulatory SNP influence or TF effects. In order to elucidate binding sites for TFs involved in the regulation of corresponding genes and their potential modification by promoter SNPs, we will apply a detailed bioinformatic analysis. For the verification of aberrant TF activation due to presence of allelic promoter variants, we will carry out luciferase reporter gene assays and chromatin immunoprecipitation assays (ChIP). Furthermore, given the potential impact of seizure frequency on gene expression, we will analyze the correlation of gene expression levels in surgical hippocampi from TLE patients with clinical or functional parameters such as gender, antiepileptic drug treatment, age at seizure onset and seizure frequency.

Adressing these objectives will provide information concerning the functional impact of transcriptional control modules and allelic promoter variants relevant in distinct pathogenetic aspects of neurological disorders. This may support the development of novel treatment strategies for episodic diseases of the CNS by antagonizing transient pathogenetic promoter activation mechanisms and by interfering with such transcriptional cascades to provide new perspectives for anticonvulsive therapies.

3 Promoter variants determine γ-aminobutyric acid homeostasis-related gene transcription in human epileptic hippocampi

3.1 Introduction

Clinical and experimental evidence demonstrates epileptic seizures as a consequence of neuronal hyperexcitability resulting from a chronic imbalance between excitation and inhibition in the brain (Feng et al., 2008). Gamma-aminobutyric acid (GABA) represents the predominant inhibitory neurotransmitter in the adult brain (Krnjević and Schwartz, 1967). GABA stimulates both metabotropic and ionotropic $GABA_A$ receptors. The $GABA_A$ receptor belongs to the superfamily of ligand-gated ion channels which mediate the majority of rapid inhibitory neurotransmission in the central nervous system (Mody and Pearce, 2004; Steiger and Russek, 2004). The binding of GABA to the $GABA_A$ receptor results in chloride influx leading to inhibitory postsynaptic currents (Mody and Pearce, 2004). Commonly, $GABA_A$ receptors are composed of five from at least 18 known subunits (α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3) (Frugier et al., 2007). Several gene clusters encode for these subunits, such as the *GABRA5*, *GABRB3* and *GABRG3* gene cluster residing on chromosome 15q11-12 and encoding for the α5, β3 and γ3 subunits (Glatt et al., 1997). Several neurological and psychiatric diseases are known to be associated with changes in $GABA_A$ receptor expression. Alterations in $GABA_A$ receptor subunit composition have been reported in TLE patients with hippocampal sclerosis (Loup et al., 2000).

The GABA β3 subunit has gained our special attention since it plays a role in several epileptic disorders. As previously reported, it shows a prevalent expression in prenatal brain regions (Laurie et al., 1992) and is encoded by the *GABRB3* gene. Furthermore, Urak *et al.* identified the SNP rs4906902 in the promoter region of *GABRB3* to be over-

represented in childhood absence epilepsy (CAE) (Urak et al., 2006). The importance of *GABRB3* in epilepsy is underlined by a *GABRB3* knockout mouse model. Disruption of *GABRB3* results in electroencephalographic abnormalities including seizures (DeLorey et al., 1998).

GABA homeostasis is based on a multilayered and complex system containing several relevant interacting components. Here, the enzyme succinic semialdehyde dehydrogenase (SSADH) is involved in the catabolism of GABA. After conversion of GABA to succinic semialdehyde by GABA transaminase, SSADH catalyzes succinic semialdehyde to succinic acid (Blasi et al., 2002). The impairment of SSADH leads to deficits in GABA degradation and accumulation of succinic semialdhehyde that is converted to γ-hydroxybutyric acid (GHB). Previous studies suggested that GHB influences multiple neurotransmitter systems like dopamine, serotonin, acetylcholine and GABA by passing through the blood-brain barrier (Maitre, 1997; Nava et al., 2001; Crunelli et al., 2006). Considering the relevance of GABA homeostasis in epileptic phenotypes, increased levels of GHB were suggested to contribute to the emergence of seizures (Snead, 1991; Wong et al., 2003).

The genetic locus for SSADH, *ALDH5A1*, is located near the tentative susceptibility loci for juvenile myoclonic epilepsy (JME) (Sander et al., 1997). Intriguingly, a two-marker haplotype with a trinucleotide repeat polymorphism (rs1883415-TNR) is detected in the promoter region of *ALDH5A1* (Lorenz et al., 2006). This polymorphism is associated with JME and idiopathic generalized epilepsy (IGE). Also, the knockout mouse model of *ALDH5A1* develops seizure disorders progressing from absence seizures to lethal status epilepticus (SE) (Cortez et al., 2004).

Here, we focused on two potentially *cis*-acting SNPs located in the promoter regions of GABA homeostasis-relevant genes. While the SNP rs4906902 identified in the promoter region of *GABRB3* is known to be associated with CAE, the SNP rs1883415 in the promoter region of *ALDH5A1* is associated with JME and IGE. In order to determine the potential impact of those promoter polymorphisms to relevant gene expression levels,

we used the access to human surgical brain tissue of TLE patients. We first stratified TLE patients according to their corresponding SNP genotypes. Subsequently, we utilized a combinatorial approach including molecular genetic, bioninformatic and *in vitro* studies to examine the effects of SNP rs4906902 and SNP rs1883415 in modified gene transcription and binding affinity of TFs.

My contribution to this work comprises the sample slice preparation, DNA and mRNA isolation, genotyping experiments of candidate SNPs, real-time reverse transcription-polymerase chain reaction experiments, generation of reporter plasmid constructs for transient transfections in cell culture and subsequent luciferase assay experiments as well as the bioinformatic, statistical analyses and writing of the manuscript.

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ORIGINAL ARTICLE

Promoter Variants Determine γ-Aminobutyric Acid Homeostasis-Related Gene Transcription in Human Epileptic Hippocampi

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Abstract

The functional consequences of single nucleotide polymorphisms associated with episodic brain disorders such as epilepsy and depression are unclear. Allelic associations with generalized epilepsies have been reported for single nucleotide polymorphisms rs1883415 (*ALDH5A1*; succinic semialdehyde dehydrogenase) and rs4906902 (*GABRB3*; $GABA_A$ β3), both of which are present in the 5′ regulatory region of genes involved in γ-aminobutyric acid (GABA) homeostasis. To address their allelic association with episodic brain disorders and allele-specific impact on the transcriptional regulation of these genes in human brain tissue, DNA and messenger RNA (mRNA) isolated from hippocampi were obtained at epilepsy surgery of 146 pharmacoresistant mesial temporal lobe epilepsy (mTLE) patients and from 651 healthy controls. We found that the C allele of rs1883415 is accumulated to a greater extent in mTLE versus controls. By real-time quantitative reverse transcription–polymerase chain reaction analyses, individuals homozygous for the C allele showed higher *ALDH5A1* mRNA expression. The rs4906902 G allele of the *GABRB3* gene was overrepresented in mTLE patients with depression; individuals homozygous for the G allele showed reduced *GABRB3* mRNA expression. Bioinformatic analyses suggest that rs1883415 and rs4906902 alter the DNA binding affinity of the transcription factors Egr-3 in *ALDH5A1* and MEF-2 in *GABRB3* promoters, respectively. Using in vitro luciferase transfection assays, we observed that, in both cases, the transcription factors regulate gene expression depending on the allelic variant in the same direction as in the human hippocampi. Our data suggest that distinct promoter variants may sensitize individuals for differential, potentially stimulus-induced alterations of GABA homeostasis-relevant gene expression. This might contribute to the episodic onset of symptoms and point to new targets for pharmacotherapies.

Key Words: Egr-3, Epilepsy, Human brain tissue, Luciferase, MEF-2, Promoter.

INTRODUCTION

Many severe brain disorders, including epilepsy and depression, manifest with episodic rather than permanent or prolonged symptoms (1, 2). Alterations of neuronal function in affected individuals due to mutations within genes related to excitability and neurotransmission, including ion channels, are well known (3–5). However, such familial mutational channelopathies generally affect few patients with high penetrance. Recent data suggest that quantitatively acquired changes of gene expression, that is, in transcriptional channelopathies, are critically involved in the pathogenesis of episodic brain diseases (6–8). Several susceptibility variants, particularly in the promoter region, have been identified in patients experiencing these neurologic disorders; these results suggest that alterations of gene expression mediate pathogenetic effects (9).

Epilepsy surgery provides seizure control in most patients with pharmacoresistant mesial temporal lobe epilepsy (mTLE) (10), and hippocampal specimens from these procedures provide the opportunity for studying the effects of promoter-associated single nucleotide polymorphisms (SNPs) on the messenger RNA (mRNA) expression of corresponding genes in the brain tissue. Because alterations of γ-aminobutyric acid (GABA)-ergic inhibition are highly relevant to epilepsy and depression (11–14), we focused on the functional role of 2 potentially *cis*-acting SNPs in the promoters of GABA homeostasis-relevant genes for transcription. To this end, we stratified mTLE patients according to their genotypes for expression analyses; this strategy overcomes the nonavailability of control (i.e. without epilepsy), human hippocampal surgical specimens.

The SNP rs1883415 (A/C) is located 3,750 bp upstream of the transcription start site of the gene *ALDH5A1* that encodes the aldehyde dehydrogenase 5 family member A1. The minor allele (C) frequency in a white population represents 34% (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A282). The A allele of this polymorphism, in the context of a 2-marker haplotype together with a trinucleotide repeat $(TAA)_{11}$ allele, was

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previously reported as having a significant association with idiopathic generalized epilepsy (IGE) and juvenile myoclonic epilepsy (JME) (15).

The exon 1a promoter region of the $GABA_A$ β3 subunit (*GABRB3*) gene comprises an A/G polymorphism (rs4906902) 897 bp upstream of the start site of *GABRB3*. The minor allele G is found with a frequency of 22% in a white population according to the dbSNP database (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A282) and is overrepresented in the absence of epilepsy in childhood (CAE) (16). *GABRB3* is also linked to depression (4, 17).

Initial studies of rs1883415 in *ALDH5A1* associated with IGE and JME, and those of rs4906902 in *GABRB3* associated with CAE (15, 18), have not been replicated. However, there is inevitably a high false-positive rate of candidate genetic variants and traits, and only few reported associations with such complex epilepsy forms have been replicated (19). Distinct pathomechanisms may be related to different forms of epilepsy syndromes; therefore, the failure to replicate reported associations does not necessarily disprove the existence of a genetic susceptibility. Because mTLE shares the feature of transient onset of hyperexcitability with IGE, JME, and CAE, different genetic variants may play different roles in mTLE. Here, using a novel combination of molecular genetic, bioinformatic, and in vitro molecular biologic approaches, we investigated the potential roles of rs1883415 and rs4906902 SNPs in impaired gene transcription and transcription factor (TF) binding in episodic brain disorders.

MATERIALS AND METHODS

Patient Criteria and Surgical Specimens

Biopsy specimens were obtained from 146 white patients with chronic pharmacoresistant mTLE who underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center (20). Presurgical evaluation using a combination of noninvasive and invasive procedures revealed that seizures originated in the mesial temporal lobe in all patients (21). Surgical removal of the hippocampus was clinically indicated in every case. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University of Bonn Medical Center. Informed written consent was obtained from all patients. Comprehensive clinical characteristics of patients were available for our analyses (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A283).

Symptoms of depression in the mTLE patients were assessed according to the Beck Depression Inventory (BDI; threshold BDI ≥12) by experienced psychiatrists at the University of Bonn Neurocenter, and groups of patients with and without depression were identified (22). It is possible that patients with mTLE who had not developed depression will develop it, but the symptoms had not occurred in the ''mTLE patients without depression'' group to the time point of this study. Individuals were followed up for several years because of their pharmacoresistant mTLE.

All tissue samples were from identical regions of the hippocampus. Fresh-frozen sections were analyzed according to international standards and carefully matched for anatomic preservation by experienced neuropathologists (A.J.B. and P.N.) (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A283). Up to five 20-μm-thick tissue sections were used for DNA and mRNA isolation.

DNA Isolation and SNP Genotyping Analysis

DNA was isolated from tissue specimens using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol (23). Additional blood DNA samples were obtained from mTLE patients with depression before epilepsy surgery (n = 56). Genotyping was performed with TaqMan SNP Genotyping Assays (*ALDH5A1* rs1883415: C__2479666_1 and *GABRB3* rs4906902: C__11300465_10) (Applied Biosystems, Foster City, CA) on an ABI Prism 9700HT sequence detection system (PE Applied Biosystems). Allelic discrimination was carried out using the SDS 2.2 software.

RNA and Complementary DNA Preparation

mRNA was isolated as previously described using the Dynabeads mRNA Direct Micro Kit (Dynal, Oslo, Norway) according to the manufacturer's protocol (24). Complementary DNA (cDNA) was synthesized by reverse transcription of total mRNA using the RevertAid First-Strand cDNA Synthase Kit (Fermentas, St. Leon-Rot, Germany) following the manufacturer's protocol.

Real-Time Reverse Transcription–Polymerase Chain Reaction

ALDH5A1, *GABRB3*, and *synaptophysin* were quantified by real-time reverse transcription–polymerase chain reaction (PCR) using TaqMan Gene Expression Assays (*ALDH5A1*: Hs00542449_m1; *GABRB3*: Hs00241459_m1; Applied Biosystems). *Synaptophysin* was used as endogenous reference gene for normalization of the analyzed mRNAs as described (6, 25). We used the ABI Prism 9700HT sequence detection system (PE Applied Biosystems) and the relative $\Delta\Delta C_t$ quantification paradigm (6).

Reporter and Other Plasmids

The reporter plasmid under control of the *ALDH5A1* promoter containing the polymorphism rs1883415 was constructed in 2 steps. First, a 566-bp fragment, bioinformatically identified as potential core promoter upstream of the transcription start site of *ALDH5A1*, was amplified by PCR using human genomic DNA as template (cloned Core region: FW-primer 5′-AAA GCA GCC AGG CAG CAG-3′, Rev-primer 5′-GGC GAC AGG AAA CAG G-3′), digested with *Xho*I and *Bgl*II, and cloned into the luciferase reporter vector pGL-3-basic (Promega Biotech, Madison, WI) (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A284). Subsequently, a 261-bp region encompassing the *ALDH5A1* promoter polymorphism rs1883415 was amplified, cut with *Xho*I and *Bgl*II and cloned 5′ of the core promoter in the luciferase reporter plasmid (SNP region: FW-primer 5′-ATC CAT GCA ATG TGT GCA G-3′, Rev-primer 5′-TTC TGA ACC CAT TTC TTT GG-3′). To generate a reporter plasmid under control of the GABRB3 promoter, a subregion (927 bp) of the human *GABRB3* promoter, including the SNP rs4906902, was amplified by PCR using human genomic DNA

TABLE 1. Genotype and Allele Frequencies of Candidate Single Nucleotide Polymorphisms in Mesial Temporal Lobe Epilepsy and Control Groups

					Genotype Frequency				Allele Frequency			
SNP	**Gene**	**Group**	**n**	**AA**	**AC**	**CC**	**χ^2**	**p**	**A Allele**	**C Allele**	**χ^2**	**p**
rs1883415	*ALDH5A1*	mTLE	140	0.264 (37)	0.572 (80)	0.164 (23)	12.59	0.0019	0.55 (154)	0.45 (126)	8.936	0.0028
		Control*	651	0.425 (277)	0.439 (286)	0.135 (88)			0.645 (840)	0.355 (462)		
SNP	**Gene**	**Group**	**n**	**AA**	**AG**	**GG**	**χ^2**	**p**	**A Allele**	**G Allele**	**χ^2**	**p**
rs4906902	*GABRB3*	mTLE	142	0.669 (95)	0.296 (42)	0.035 (5)	1.2	0.5498	0.817 (232)	0.183 (52)	0.01	0.9918
		Control†	561	0.655 (366)	0.324 (181)	0.021 (12)			0.183 (205)	0.817 (913)		

Candidate SNPs were described by name of corresponding gene, rsID, and the genotype and allele frequency in mTLE and control groups. Absolutes numbers are shown in parentheses. χ^2 test and 2-tailed p value.

None of the genotype distributions in the controls deviated significantly from those expected by Hardy-Weinberg equilibrium.

*Control according to Lorenz et al (15).

†Control according to Urak et al (16).

mTLE, mesial temporal lobe epilepsy; rsID, reference SNP identifier; SNPs, single nucleotide polymorphisms.

as template (FW-primer 5′-ATC TTT CAGG TAC TGC GGT CA-3′, Rev-primer 5′-CTC CGA GCA GCC AAA CG-3′) (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A284), cut with *Xho*I and *Bgl*II and cloned into pGL-3-basic.

Transient Transfections

NG108-15 neuroblastoma cells were plated at a density of 80% confluence in 48-well plates and grown in 0.5 mL of Dulbecco modified Eagle medium supplemented with 10% vol/vol fetal calf serum, 5% Pen Strep, and 5% HAT. Transfection of the cells was carried out using lipofectamine (Invitrogen, Darmstadt, Germany), following the manufacturer's protocol. For each well of the 48-well tissue culture plate, 50 ng of luciferase reporter plasmid, 12.5 ng of pRL-TK or 12.5 ng of RL-SV40, and 0.5 μL of lipofectamine were mixed with 25 μL of medium. The mixture was incubated for 20 minutes at room temperature and then added to the appropriate wells. Cells were grown in culture medium for 6 to 12 hours at 37°C and 5% CO_2. Thereafter, the medium was replaced by fresh medium, and the cells were used for experiments 48 hours after transfection.

Luciferase Assay

Renilla luciferase was used to normalize the transfection efficiency data, and a Dual Luciferase Reporter Assay System was used according to the manufacturer's specifications (Promega). *Renilla* and *Firefly* luciferase activities were determined using the Glomax Luminometer (Promega), counting each sample 4 times. The results are given as *Firefly*/*Renilla* relative light units (if not otherwise indicated).

Bioinformatic Analyses

The Web and software tools CpGPlot (26), Promoter2.0 (27), COMET (28), and Eponine (29) were used to identify potential promoter regions upstream of a gene of interest. To identify the position and conservation of potential TF binding sites, the software tool PoSSuMsearch (30) using position-specific-scoring matrices from the TRANSFAC database (31, 32) was applied to the potential promoter regions. For filtering the potential TF binding sites, a motif similarity score (MSS) of 80% was applied.

Statistical Analyses

χ^2 analysis was used to test significance of differences in allele and genotype frequencies in controls versus affected subjects. A 2-sided type 1 error rate of $p = 0.05$ was chosen for the analyses. No correction for multiple testing was performed with respect to the exploratory design and modest statistical power of this study. Analysis of variance *t*-test was used as indicated to evaluate the statistical significance of the TaqMan and luciferase results.

TABLE 2. Genotype and Allele Frequencies of Candidate Single Nucleotide Polymorphisms in "Mesial Temporal Lobe Epilepsy With Depression" Versus "Mesial Temporal Lobe Epilepsy Without Depression" Groups

						Genotype Frequency				Allele Frequency			
SNP	**Gene**	**Group**	**Depression**	**n**	**AA**	**AC**	**CC**	**χ^2**	**p**	**A Allele**	**C Allele**	**χ^2**	**p**
rs1883415	*ALDH5A1*	mTLE	+	76	0.276 (21)	0.368 (28)	0.355 (27)	13.32	0.0013	0.461 (70)	0.539 (82)	1.348	0.2457
		mTLE	−	110	0.1 (11)	0.6 (66)	0.3 (33)			0.4 (88)	0.6 (132)		
SNP	**Gene**	**Group**	**Depression**	**n**	**AA**	**AG**	**GG**	**χ^2**	**p**	**A Allele**	**G Allele**	**χ^2**	**p**
rs4906902	*GABRB3*	mTLE	+	81	0.691 (56)	0.198 (16)	0.111 (9)	7.18	0.0276	0.79 (128)	0.21 (34)	0.112	0.7373
		mTLE	−	107	0.598 (64)	0.355 (38)	0.047 (5)			0.776 (166)	0.224 (48)		

Candidate SNPs were described by name of corresponding gene, rsID, and the genotype and allele frequency in mTLE and control dbSNP group. Absolutes numbers are shown in parentheses. χ^2 test and 2-tailed p value.

−, absent; +, present; mTLE, mesial temporal lobe epilepsy; SNPs, single nucleotide polymorphisms.

RESULTS

Distribution of Allelic Variants in Different Patient Groups

We first examined the allele and genotype frequencies of the *ALDH5A1* and *GABRB3* SNPs in 146 mTLE patients and a German population control group (Table 1) (15, 18). Association analysis for the *ALDH5A1* promoter SNP rs1883415 revealed a significant excess of the C allele in 140 mTLE patients versus controls ($\chi^2 = 8.94$, $df = 1$, $p = 0.0028$). Likewise, the genotypic frequencies differed significantly between mTLE patients and controls ($\chi^2 = 12.59$, $df = 2$, $p = 0.0019$; Table 1).

For the *GABRB3* promoter SNP rs4906902, we did not observe a significant increase of the A allele in 142 mTLE patients compared with the 561 German population controls ($\chi^2 = 0.01$, $df = 1$, $p = 0.99$). The genotype frequencies were not significantly different ($\chi^2 = 1.2$, $df = 2$; $p = 0.55$; Table 1).

Because neuropsychiatric (particularly depressive) symptoms are frequent in mTLE patients, we further tested a comorbidity phenotype by stratifying the patients according to the presence or absence of depression. We also increased our sample population with blood DNA samples from additional mTLE patients with depression (n = 56; BDI ≥12). We genotyped these patients for the 2 respective candidate SNPs (Table 2). The genotype frequency in mTLE patients with depression versus mTLE patients without depression was significantly different for the *ALDH5A1* promoter SNP rs1883415 ($\chi^2 = 13.32$, $df = 2$, $p = 0.0013$), that is, the C allele genotype is significantly increased in patients with symptoms of depression (Table 2).

Intriguingly, for the SNP rs4906902 located in the *GABRB3* promoter, our analysis demonstrated that, in contrast to the entire TLE patient collective, TLE patients with depressive symptoms displayed a significant increase of the G/G genotype compared with TLE patients without depression ($\chi^2 = 7.18$, $df = 2$; $p = 0.0276$; Table 2).

We did not correct for multiple testing in this association analysis. Altogether, we performed 4 (partially dependent) tests comparing the genotypic distribution of 2 common SNPs in 2 phenotype models (mTLE vs controls, mTLE with depression vs mTLE without depression). In total, 3 of 4 tests revealed associations at a nominal p value of 0.05. Considering the exact p values, the significance level of each association can be adjusted for multiple testing according to the individual perspective. For the expression quantitative trait loci (eQTL) analyses, we carried out a post hoc exploration to examine whether the observed association of the SNP genotype and mRNA transcript levels might be spurious due to confounding by several clinical variables that might affect gene expression, for example, age-of-onset, sex, and age at sampling. These additional tests were not part of the original study hypothesis and, therefore, were not considered as additional tests requiring a p value adjustment. They did not result in significant correlations of distinct genotypes to other clinical variables (Table, Supplemental Digital Content 4, http://links.lww.com/NEN/A285). These experiments demonstrate intriguing correlations of the genetic variability of *ALDH5A1* and *GABRB3* to mTLE and depression.

mRNA Expression of *ALDH5A1* and *GABRB3* Is Affected by the Promoter Polymorphisms

Next, we analyzed whether and how the presence of allelic variants in respective promoters influences the mRNA expression of *ALDH5A1* or *GABRB3* in human hippocampal brain tissue derived from pharmacoresistant mTLE patients during epilepsy surgery (Figs. 1A, B). We found that the relative gene expression of *ALDH5A1* mRNA in mTLE patients homozygous for the A allele (n = 18) was significantly decreased compared with the group of mTLE patients homozygous for the C allele (n = 19; Fig. 1C; *t*-test: *, $p = 0.037$).

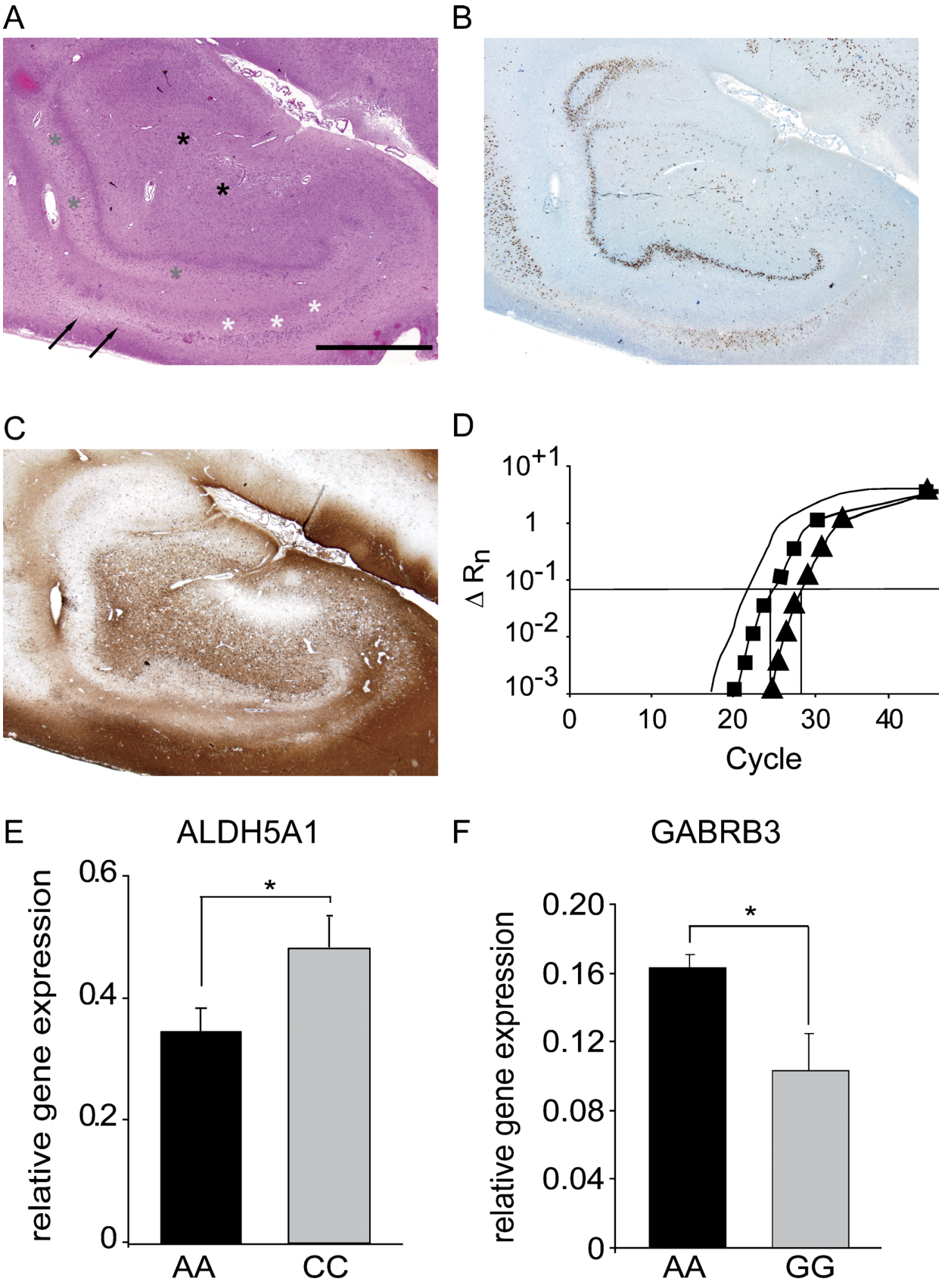
The relative expression of *GABRB3* mRNA was significantly increased in the mTLE patient group with the A/A genotype (n = 52) compared with hippocampi of mTLE patients carrying the G/G genotype (n = 4; *t*-test: *, $p = 0.049$; Fig. 1D). Importantly, neither different basic neuropathology patterns nor clinical variables covaried with the differences in gene expression observed (Table, Supplemental Digital Content 4, http://links.lww.com/NEN/A285).

Because antiepileptic drugs may have effects on gene expression and cause mood disturbances, we analyzed the effects on gene expression of antiepileptic as well as antidepressant drugs (Table, parts A and B, Supplemental Digital Content 5, http://links.lww.com/NEN/A286). We did not find significant differences in gene expression according to treatment with antiepileptic/antidepressant compounds. Furthermore, we observed no significant overrepresentation of antiepileptic pharmacotherapy in mTLE patients with depression (Table, part C, Supplemental Digital Content 5, http://links.lww.com/NEN/A286). Thus, our data suggest that polymorphisms in the promoter region of the analyzed target genes can affect corresponding gene expression independent of the variables examined.

Promoter SNPs Influence the Functionality of Transcription Factors Regulating Expression of *ALDH5A1* or *GABRB3*

To examine further whether the above described SNPs directly affect the DNA binding affinity (and thereby functionality of transcription factors), we searched for TF binding sites whose MSS was significantly altered by the presence of the SNP. For rs1883415 in the *ALDH5A1* promoter, we predicted that the TF binding sites for Egr-3 would exhibit a higher MSS (74%) in the SNP sequence variant compared with the wild-type promoter allele (MSS = 62%; Fig. 2A). Therefore, the effect of the 2 *ALDH5A1* alleles on basal transcription and Egr-3–stimulated transcription was determined by measuring *Firefly* luciferase 48 hours after transfection. The results showed no significant difference in basal expression between the 2 alleles. By contrast, whereas overexpression of Egr-3 did not induce expression of the C allele, there was a statistically significant increase in rs1883415 allele expression after Egr-3 stimulation when compared both to the unstimulated C allele (1.7-fold; *t*-test: **, $p = 0.0028$; n = 4 each), as well as to the stimulated A allele (*t*-test: , *, $p = 0.025$; n = 4 each; Fig. 2C).

In the promoter region of *GABRB3*, we detected a potential binding site for the activating TF MEF-2 with a higher binding affinity to the A allele (74%) than to the G allele (66%; Fig. 2B). The DNA binding affinity of the previously described



N-Oct-3 binding site was not affected by the presence of the polymorphism (80% binding affinity in the A-variant and 81% in the G-variant [16] and data not shown). We did not observe an effect of the rs4906902 allelic variants on basal promoter activity. Interestingly, exposure to increased MEF-2 levels resulted in a significant activation of the A/A genotypic promoter variant (Fig. 2D; 1.8-fold; *t*-test: ***, p = 0.0009; n = 4 each) but not of the G/G variant (Fig. 2D; n = 4 each).

DISCUSSION

Our data demonstrate that the promoter-localized SNPs rs4906902 and rs1883415 substantially affect transcription levels of the corresponding GABA homeostasis-relevant genes in human brain tissue by putatively altering the binding affinity of corresponding TFs. Several limitations of the data need to be considered with respect to their interpretation. In the genetic analyses carried out in this study, the number of patients available for analyses did not allow us to replicate a genetic association with mTLE. However, in contrast to genetic analyses alone, in concurrent genetic and expression analyses based on fresh-frozen patient hippocampal tissue, the number of patients is (for obvious reasons) more limited. Furthermore, genome/transcriptome-wide analyses show a large number of eQTLs. However, it should be noted that eQTL data derived from the human brain are limited and have been obtained mainly from postmortem tissue samples that have certain restrictions with respect to mRNA conservation (33–35); moreover, comparable eQTL analyses for bioptic human brain tissues are not available. Therefore, our approach (although certainly not genome-wide) concentrated on genes selected according to biologic plausibility.

Distinct statistically significant associations of rs4906902 allelic variants in the *GABRB3* promoter have been reported in episodic brain disorders. The minor allele G is overrepresented in childhood absence epilepsy (16), whereas the major allele A of *GABRB3* is linked to depression in patients with posttraumatic stress disorder (17). Other data demonstrate that the *GABRB3* exon 1a SNP G allele does not differ significantly between CAE patients and controls (15, 18). Here, we found that the G allele was significantly more frequent in mTLE patients with depression (Tables 1 and 2). Generally, genetic variation in *GABRB3* substantially influences the risk for bipolar disorders (4), and there is apparently no overall abundance of one particular rs4906902 allele in episodic brain disorders. On the contrary, both individual alleles are differentially overrepresented in patients in whom depressive mood impairment has its onset in distinct pathologic backgrounds, that is, the A allele in posttraumatic stress versus the G allele in epilepsy. This argues that depressive symptoms manifest in diverse contexts by the contribution of different, potentially even opposite molecular alterations.

To understand the functional consequences of the presence of these allelic variants, we investigated whether such genetic variation affects mRNA transcription. In brain tissue stratified according to the genotype, we observed substantially different *GABRB3* expression levels dependent on the rs4906902 genotype, that is, a significantly lower expression of *GABRB3* mRNA in hippocampi of mTLE patients homozygous for the G allele compared with those homozygous for the A allele (Fig. 1D). Reduced availability of the β3 subunit of the $GABA_A$ receptor has been shown to be epileptogenic, that is, its deletion in mice leads to severe behavioral deficits and epilepsy (36). $GABA_A$ receptor–mediated miniature inhibitory postsynaptic currents in cortical neurons in cultures from $\beta3^{-/-}$ mice are faster than of littermate controls and more prolonged by zolpidem (36). However, in human mTLE hippocampi, increased mRNA and protein levels have been observed for the $GABA_A$ receptor β3 subunit in the dentate molecular layer and in the subiculum (37). These data suggest distinct allelic variant accumulation with opposite consequences for *GABRB3* mRNA expression in patient subgroups with epilepsy and depression. With respect to promoter control in the region of rs4906902, our bioinformatic prediction suggested substantially differential binding for MEF-2, with a higher affinity for the A allele. Similar to previous data, we found a tendency to increased basal activity of the G allele *GABRB3* promoter variant (16), which was, however, not significant. Stimulation of the *GABRB3* promoter with MEF-2 revealed a substantial activation of the A allele, whereas the G allele showed virtually no promoter activation (Fig. 2). However, after pilocarpine-induced status epilepticus in rats, there is reduced hippocampal expression of MEF-2 mRNA compared with controls (unpublished data). These data would be compatible with a generally attenuated activation of the *GABRB3* promoter in mTLE hippocampi. Interestingly, MEF-2 target genes have diverse functions at synapses, and several of them are linked to epilepsy and depression (38). Considering $GABA_A$ β3 as an active component of ligand-gated ion channels, our data on rs4906902

FIGURE 1. Analysis of relative gene expression of candidate genes *ALDH5A1* and *GABRB3* in human hippocampal tissue. **(A)** Hematoxylin and eosin staining shows the characteristic pattern of Ammon horn sclerosis (AHS) in a hippocampal biopsy specimen after epilepsy surgery of a pharmacoresistant mesial temporal lobe epilepsy (mTLE) patient (gray asterisk, dentate gyrus [DG] granule cell layer; black asterisk, CA4; white asterisk, CA2; black arrows, CA1). **(B)** There is segment neuronal cell loss most pronounced in CA4 and CA1; CA2 and DG are well preserved (NeuN immunohistochemistry). **(C)** There is marked astrogliosis in the hippocampal formation (glial fibrillary acidic protein immunohistochemistry). **(D)** Quantitative determination of candidate gene mRNAs was carried out using a TaqMan approach. In a representative trace, the increase of fluorescence intensity demonstrating the specific amplification is shown for the *ALDH5A1* wild-type (triangles), *ALDH5A1* single nucleotide polymorphisms (SNP) (squares) and reference (black curve) mRNAs. The horizontal line marks the point of the polymerase chain reaction in the exponential stage where ''threshold cycles'' are determined for quantification of target genes. **(E)** For *ALDH5A1*, the relative gene expression in patients homozygous for the wild-type allele A (n = 18; 0.35 ± 0.04; black bar) is significantly lower compared with that for the group of mTLE patients homozygous for the SNP C (n = 19; 0.48 ± 0.05; *t*-test: *, p = 0.037; light gray bar). **(F)** Relative expression of the *GABRB3* mRNA in the group of patients homozygous for the wild-type allele A (n = 52; 0.16 ± 0.01; black bar) is significantly higher compared with that in the group of patients homozygous for the SNP allele G (n = 4; 0.1 ± 0.02; *t*-test: *, p = 0.049; light gray bar). Scale bar = **(A–C)** 2.0 mm.

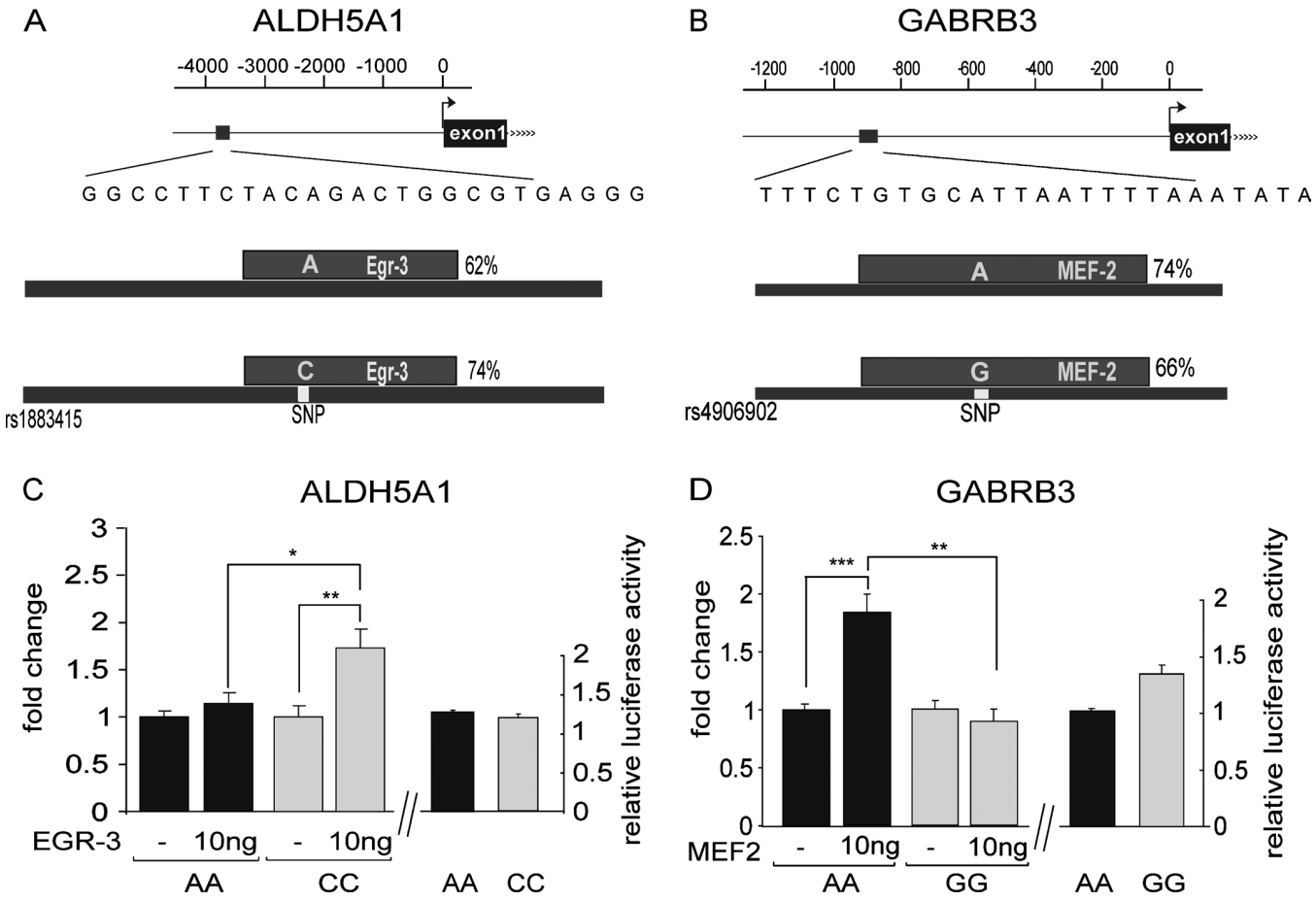


FIGURE 2. Analysis of transcription factor (TF) binding site modifications and relative luciferase activity in promoter regions of candidate genes. **(A)** The single nucleotide polymorphism (SNP) rs1883415 is located in the promoter region of the *ALDH5A1* gene 3,722 bp upstream of its transcription start site. The genomic sequence flanking the SNP contains potential TF-binding sites, characterized by different matrix similarity scores between the wild-type and SNP sequence variants. Egr-3, an ''activating'' TF, binds with a higher affinity (74%) to the SNP sequence compared with the wild-type promoter allele (62%). **(B)** The upstream region of *GABRB3* contains a SNP (rs4906902) located 841 bp upstream of exon 1 of *GABRB3*. This SNP is located within a potential binding site for the ''activating'' TF MEF-2 (large box), which is predicted to bind with a higher binding affinity to the wild-type variant (74%) than the SNP allele (66%). **(C)** The increase of the promoter activity of the *ALDH5A1* fragment homozygous for the SNP allele is significant after exposing the promoter to Egr-3 (10.0 ng of plasmid; 1.7-fold; *t*-test: **, p = 0.0028; n = 4 each; light gray bars). The wild-type promoter fragment shows no significant difference in fold change activity after the addition of Egr-3 expression plasmid compared with basal controls (n = 4 each; black bars). There is substantially stronger activation of the promoter fragment carrying the SNP compared with wild-type after exposure of both genotype promoters to Egr-3 (*t*-test: *, p = 0.025; n = 4 each). There was no significant difference in the relative luciferase activity of the basal promoters. **(D)** The activity of the *GABRB3* promoter fragment homozygous for the wild-type allele is significantly increased in fold change after exposure to MEF-2 (10 ng of expression plasmid; 1.8-fold; *t*-test: ***, p = 0.0009; n = 4 each; black bars). The promoter fragment containing the SNP allele did not show significant activity alterations after exposure to MEF-2 (n = 4 each; light gray bars). There was substantially stronger promoter activity for the wild-type than the SNP variant exposed to MEF-2 (*t*-test: **, p = 0.007; n = 4 each). The relative luciferase activity of the basal promoter fragments did not differ between the genotypes.

provide an intriguing example of a transcriptional channelopathy in the human brain that can contribute to the pathogenesis of CNS disorders.

In contrast to the immediate effects on neurotransmission by $GABA_A$ β3, *ALDH5A1* encodes the succinic semialdehyde dehydrogenase, a protein that catalyzes a critical step in the recycling and degradation of GABA (39, 40). Its deficiency results in a rare autosomal-recessive heritable disorder with prominent seizures; there are also seizures in a murine model of this deficiency (41). One of the *ALDH5A1* rs1883415 alleles for which we stratified patients here (i.e. the A allele) is significantly accumulated in IGE and JME (15). Here, we consistently observed a reduced expression of *ALDH5A1* mRNA in the brain tissue of patients homozygous for this (Fig. 1C). This finding is in line with a reduced expression of the succinic semialdehyde dehydrogenase and the hypothesis of a lower turnover of GABA, which may at least partially parallel the functional consequences of a complete lack of the succinic

semialdehyde dehydrogenase in patients with genetic deficiency of the molecule. Conversely, we observed abundance of the *ALDH5A1* C allele in the entire mTLE series, as well as in mTLE patients with depression.

These data suggest that proper availability of the succinic semialdehyde dehydrogenase and ''fine-tuning'' of the homeostasis of GABA degradation are critical for adequate neurotransmission; not only reduced levels of the semialdehyde dehydrogenase but also its increased levels may impair neuronal function in a way that contributes to different clinical manifestations including mood disorders and focal seizures. Unfortunately, we did not have sufficient amounts of patient fresh-frozen brain tissue available to carry out comparisons of *ALDH5A1* mRNA expression in mTLE patients with and without depressive symptoms. With respect to potential promoter control mechanisms, we identified a highly conserved Egr-3 binding site located at the rs1883415 SNP. Intriguingly, the C allele shows stronger promoter activation after stimulation with Egr-3. Egr-3 mRNA is increased in the hippocampal formation after status epilepticus induced by kainic acid in a rat temporal lobe epilepsy model (42). These data would be compatible with an increased stimulus by Egr-3 on the expression of the respective gene in individuals harboring the *ALDH5A1* rs1883415 C allele. Furthermore, Egr-3 has also been associated with the control of the expression of the α4 subunit of the $GABA_A$ receptor (43). Our present data suggest that, in human mTLE, impairment of GABA signaling may be due not only to the dynamic expression of GABA receptor subunits themselves but also to the degradation-relevant molecules, that is, succinic semialdehyde dehydrogenase.

With respect to GABA-mediated signaling, the aberrant expression of mRNAs coding for GABA receptor subunits and GABA-degrading enzymes may combine functionally and contribute to a disturbed neurotransmission in affected brain tissue. Our data suggest a relevance of genetic promoter variants for the expression of corresponding genes in the brain tissue of patients experiencing episodic CNS disorders. In the future, genetic profiling of such variants may open the perspective to enable ''personalized'' pharmacotherapies more effective for chronic recurrent brain disorders.

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Pernhorst et al., Supplemental Table 1, Supplemental Digital Content 1:

Information of selected candidate SNPs. Reference SNP IDs (rsIDs), name of nearest gene with the corresponding distance to the TSS* in base pairs and the position of the SNP are represented. For each candidate SNP the different occurring alleles, the minor allele and the frequency of the allele in the Caucasian population (source: dbSNP) is shown. The documented location of the SNP according to dbSNP is presented as well.

rsID	Gene	SNP Positi-on	Distance to TSS*	Alleles	Minor allele	Frequency in CEU**	Location of SNP
rs1883415	ALDH5A1	chr.6, 24599454	- 3750bp	A/C	C	0.345	nearGene-5***
rs4906902	GABRB3	chr.15, 24570861	- 897bp	A/G	G	0.221	nearGene-5***

*TSS = Transcription Start Site
**CEU = Utah residents with Northern and Western European ancestry from the CEPH collection
***nearGene-5 = SNP is 5' to and 2kb upstream of gene

Pernhorst et al., Supplemental Table 2, Supplemental Digital Content 2:

Summary of patient parameters.

Parameters including gender, depression, number of seizures per month, seizure-free post-operative interval, age at seizure onset in years, age at epilepsy surgery in years, pathology and drug therapy are presented for each patient. Information on number of seizures per month relies partially on patients' information and is therefore variable (n.a.: data not available). The post-operative outcome is classified according Engel classification (class I A: completely seizure free; class I B: non disabling, simple partial seizures only; class II B: free of disabling seizures for at least 2 years; class IV B: no seizure reduction). Biopsy specimens were neuropathologically analyzed according to standard procedures and hippcampi stratified according to the pathlogical pattern of the patient into the three following groups: AHS (Ammon's horn sclerosis), Rasmussen's encephalitis and lesion associated (ganglioglioma, cavernoma, dysembryoplastic neuroepithelial tumor). Notably, also hippocampi of patients with Rasmussen's encephalitis (RE) and lesion associated TLE show substantial reactive astrogliosis in the hippocampal formation. Drug therapies do generally consist of combinations of the following compounds, i. e. Carbamazepine (CBZ), Clobazam (CLB), Lamotrigine (LTG), Levetiracetam (LEV) Oxcarbazepine (OXC), Phenytoin (PHT), Phenobarbital (PB), Pregabalin (PGB), Topiramate (TPM), Valproat (VPA), Vigabatril (VGB) and Zonisamide (ZON).

Patient	Gender	Depression	Number of seizures per month	Seizure-free Post-operative interval	Age at seizure onset in years	Age at epilepsy surgery in years	Pathology	Drug therapy
1	female	yes	1-3 CPS	I A	6	47	AHS	LEV, CBZ
2	male	no	2 CPS per day	I A	11	11	lesion associated	CBZ, LTG
3	male	no	5 CPS	I A	3	19	AHS	VPA
4	male	yes	2–4 CPS	I A	40	48	AHS	LTG, ZON
5	male	no	3-7CPS	IV B	10	45	AHS	LTG, CBZ, VPA
6	female	no	daily	IV B	1	38	AHS	VPA, LTG
7	female	no	4 CPS	IV B	20	32	AHS	n.a.
8	female	no	2-3 CPS	I A	1	35	AHS	LTG
9	male	yes	n.a.	IV B	n.a.	39	n.a.	n.a.
10	male	no	4-6 CPS	IV B	13	19	lesion associated	CBZ , VGB , CLB
11	male	n.a.	30 CPS	IV B	21	39	lesion associated	TPZ, CBZ, PHT, LEV

12	female	yes	6-8 CPS	IV B	13	46	AHS	OXC
13	male	yes	3 CPS	IV B	7	29	lesion associated	LEV, LTG, ZON, PB, PHT
14	male	no	2-3 CPS	IV B	6	45	AHS	OXC, LTG
15	female	no	3 CPS per day	I A	1	10	AHS	CBZ, VPA
16	male	no	2 CPS	I A	5	8	AHS	n.a.
17	female	yes	3 CPS	IV B	6	n.a.	AHS	n.a.
18	male	no	3-4 CPS	IV B	18	28	AHS	LTG, CLB
19	female	yes	3-10 CPS	I A	18	44	AHS	OXC, LTG, GBP
20	male	yes	3 CPS	IV B	46	58	AHS	LTG, LEV
21	female	yes	3-4 CPS	IV B	n.a.	36	AHS	LTG, CBZ
22	female	no	2 CPS	IV B	12	20	AHS	LEV, LTG
23	female	no	8 CPS	I B	4	50	AHS	VPA
24	female	no	3 CPS	IV B	3	24	lesion associated	VPA, CBZ
25	female	yes	2 CPS	I A	n.a.	29	AHS	CBZ, LTG
26	male	yes	6 CPS	I A	3	24	AHS	LEV, LTG, CBZ
27	female	no	2-3 CPS	I A	13	25	AHS	LEV, CBZ
28	female	no	1-2 CPS	IV B	1	11	AHS	LEV
29	female	no	1-2 CPS	I A	1	29	AHS	LEV, OXC
30	female	no	3 CPS	I A	1	8	n.a.	LEV, PGB, CLB, MSM
31	female	yes	2 CPS	IV B	2	8	AHS	LEV
32	male	n.a.	2 CPS	n.a.	2	8	n.a.	LEV, CLB
33	male	no	2-4 CPS	IV B	10	56	AHS	ZON, VPA, OXC
34	male	yes	1 CPS	I A	30	38	AHS	LTG, PHT
35	female	no	3 CPS per day	I A	4	0	AHS	LEV, TPM, CBZ
36	female	yes	16 CPS	n.a.	13	39	AHS	OXC, LEV, LTG
37	female	no	2-4 CPS	I A	5	41	AHS	LEV, CBZ
38	male	no	3-4 CPS	IV B	19	31	AHS	VPA, CBZ
39	female	no	3-15 CPS	I A	2	24	AHS	LEV, LTG, ZON
40	male	no	1 CPS	IV B	16	56	AHS	LEV
41	male	no	15-20 CPS	I A	10	15	AHS	CBZ
42	male	no	8 CPS	I A	9	10	lesion associated	LTG
43	female	no	5 CPS per year	I A	10	19	lesion associated	CBZ
44	female	no	24 CPS	IV B	4	45	AHS	CBZ
45	male	no	33 CPS	I A	25	35	AHS	CBZ
46	male	no	20 CPS	I A	12	26	AHS	CBZ, GBP

47	female	no	n.a.	I A	7	16	lesion associated	n.a.
48	male	no	3 CPS	IV B	7	24	lesion associated	VPA
49	female	no	2 CPS	IV B	1	14	AHS	LEV
50	female	no	1 CPS per week	I A	1	42	AHS	TPM, VPA
51	female	no	2 CPS per day	n.a.	1	32	AHS	CBZ, GBP
52	male	yes	6-12 CPS	IV B	27	50	AHS	OXC, PHT
53	female	yes	2 CPS	IV B	14	22	lesion associated	LTG, LEV
54	male	no	4 CPS	IV B	5	32	lesion associated	CBZ, LTG
55	male	no	n.a.	n.a.	7	n.a.	AHS	n.a.
56	male	no	3 CPS per week	I A	44	59	AHS	LTG, TPM, CLB
57	male	n.a.	15-20 CPS	I A	1	38	AHS	CBZ, TPM
58	male	yes	4 CPS	IV B	29	37	lesion associated	LEV
59	male	no	30-40 CPS	I A	3	17	AHS	OXC, LTG
60	female	no	4 CPS	I A	3	37	AHS	CBZ, LTG
61	male	no	5-8 CPS	IV B	3	33	AHS	VPA
62	male	yes	2 CPS	I A	2	19	lesion associated	LTG, OXC, TPM
63	male	no	1-2 CPS	IV B	28	52	n.a.	LTG, LEV
64	female	no	2-3 CPS	IV B	22	54	AHS	LEV, TPM, OXC
65	female	no	11-14 CPS	IV B	20	36	lesion associated	CBZ, CLB
66	female	no	1 CPS per day	I A	0	2	lesion associated	LTG, PB
67	female	no	1-2 CPS	IV B	15	32	AHS	CBZ
68	male	no	3-5CPS	I A	8	14	AHS	LTG, ZON
69	male	no	n.a.	I B	n.a.	n.a.	lesion associated	n.a.
70	male	no	1 CPS per day	I A	2	4	AHS	LEV, LTG
71	male	no	1-2 CPS	IV B	12	31	AHS	LTG, CBZ
72	male	no	60 CPS	I A	5	10	RE	LEV, VPA, CBZ
73	male	no	2 CPS	IV B	6	45	AHS	LTG, CBZ
74	female	yes	4-5 CPS	I A	1	39	AHS	VPA, PHT, PB
75	male	no	4 CPS	n.a.	7	41	n.a.	CBZ, VPA, LTG
76	female	no	4-5 CPS	IV B	3	32	AHS	VPA, PHT
77	male	no	4-6 CPS	I A	2	24	AHS	CBZ, PGB
78	female	no	2 CPS	I A	n.a.	32	AHS	n.a.

79	female	no	7-8 CPS	I A	n.a.	35	lesion associated	LTG
80	female	no	2-3 CPS per week	I B	12	31	lesion associated	OXC
81	female	yes	4-5 CPS	IV B	2	34	AHS	CBZ, VPA
82	male	yes	0-1 CPS	I A	39	41	AHS	PHT, LTG
83	male	no	6 CPS	I A	25	30	AHS	LEV, OXC
84	female	yes	1 CPS	IV B	12	46	AHS	LTG, PB
85	male	no	2-9 CPS	I A	8	56	AHS	CBZ
86	male	no	3-10 CPS	II B	13	31	AHS	VPA, CBZ
87	female	yes	1-2 CPS per year	I A	22	28	RE	LTG
88	female	no	1 CPS per day	I A	11	25	AHS	LTG, LEV
89	female	no	2 CPS	I A	28	38	AHS	n.a.
90	female	no	3-12 CPS	IV B	15	18	AHS	LEV, OXC, PB
91	female	no	2 CPS	I A	3	34	AHS	CBZ
92	male	yes	4-8 CPS	II B	12	30	AHS	CBZ, TPM
93	male	no	1-2 CPS	I A	20	46	AHS	CBZ
94	male	no	15 CPS	I A	4	12	AHS	ZON
95	female	no	1 CPS	I A	1	37	AHS	CBZ
96	female	no	20 CPS	I A	6	43	AHS	CBZ, TGB
97	male	yes	10-20 CPS	IV B	7	71	AHS	LEV, LTG
98	male	no	2-3 CPS	n.a.	1	10	AHS	VPA, VGB, PHT, PB
99	male	yes	1 CPS per week	IV B	12	62	AHS	CBZ, TPM
100	male	no	multiple CPS	I A	12	27	AHS	LEV, CBZ
101	female	no	4 CPS	IV B	0	31	AHS	CBZ, LEV, LTG
102	female	yes	4 CPS	I A	13	52	AHS	VPA, TPM
103	male	no	2-4 CPS	IV B	9	43	AHS	OXC, TPM
104	female	no	1 CPS per week	IV B	32	34	AHS	OXC, LTG, LEV, LZP
105	female	no	weekly	IV B	1	22	AHS	PGB, CBZ
106	female	no	9-12 CPS	I A	23	31	AHS	LEV, VPA
107	male	no	n.a.	IV B	n.a.	n.a.	AHS	n.a.
108	male	no	3-9 CPS	IV B	2	38	AHS	CBZ, VPA
109	female	no	3-9 CPS	II B	1	46	AHS	CBZ, VPA
110	male	no	1 CPS per day	IV B	10	28	lesion associated	PGB, LTG

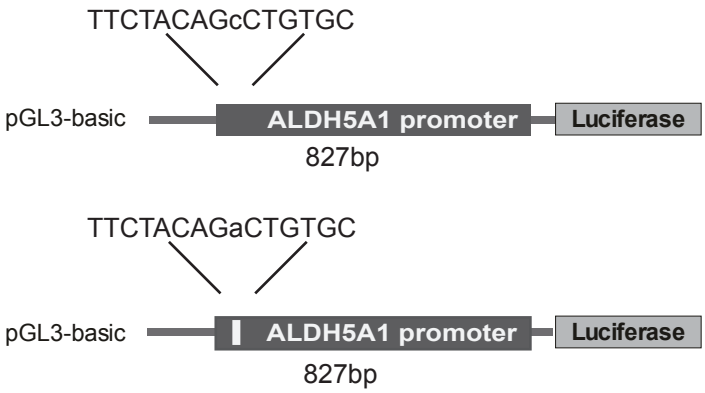
111	female	no	2 CPS	I A	8	17	AHS	TPM
112	female	no	4 CPS	I A	7	32	AHS	LTG, CBZ
113	female	no	10 CPS	I A	5	51	AHS	LEV, CBZ
114	female	no	8 CPS	I A	2	20	lesion associated	LEV, LTG
115	male	no	4 CPS per year	IV B	10	31	AHS	VPA, CBZ
116	female	no	4 CPS	I A	0	5	AHS	CBZ, LTG
117	male	no	1 CPS	II B	17	28	lesion associated	TPM, GBP
118	female	no	3 CPS	II B	17	26	AHS	VPA
119	female	yes	8 CPS	IV B	24	29	AHS	LTG
120	female	no	2-4 CPS	IV B	29	40	AHS	LTG, TPM
121	male	no	Multiple per night	I A	6	13	AHS	OXC, PRM
122	female	no	1 CPS per day	I A	8	55	lesion associated	VPA, CBZ
123	male	no	4 CPS	I A	40	60	AHS	LTG, LEV
124	male	no	40 CPS	IV B	4	42	AHS	CBZ
125	male	no	4 CPS	I A	2	19	AHS	OXC, LEV
126	male	yes	9 CPS	I A	32	39	lesion associated	LEV, TPM, CBZ
127	female	no	1 CPS	I B	5	10	AHS	VPA, PHT
128	male	no	4 CPS	IV B	11	54	AHS	LEV, CBZ
129	female	no	3 CPS per week	I A	24	60	lesion associated	CBZ, LEV
130	male	yes	1-8 CPS	IV B	3	21	AHS	CBZ, TPM
131	male	no	4-8 CPS	IV B	13	50	AHS	CBZ, CLB
132	male	yes	20-30 CPS	I B	2	34	AHS	LEV, CBZ
133	male	no	2 CPS	IV B	28	41	lesion associated	CBZ, LTG
134	female	no	2 CPS	IV B	17	32	AHS	CBZ, TPM
135	female	no	1 CPS	IV B	13	31	AHS	CBZ, LTG
136	male	no	2 CPS per day	I A	1	5	AHS	LTG
137	male	no	2-3 CPS	IV B	5	44	AHS	CBZ
138	male	no	n.a.	IV B	1	3	lesion associated	LTG, CBZ
139	male	no	2-3 CPS per day	I A	3	32	AHS	CBZ, LTG
140	female	no	2-8 CPS	I A	7	62	AHS	LEV, CBZ, PGB
141	female	no	2 CPS	IV B	11	12	lesion associated	CBZ, VGB
142	male	yes	3 CPS	IV B	3	42	AHS	CBZ
143	female	no	1-2 CPS	IV B	24	47	AHS	Cbz1200 + 2000
144	male	no	1 CPS	IV B	4	31	AHS	CBZ, GBP
145	male	no	5 CPS	I A	2	31	AHS	CBZ, LTG, CNZ

146	male	no	8 CPS	I A	13	30	AHS	LTG, CBZ
147	female	yes	2 CPS	IV B	25	57	AHS	LEV, CBZ
148	male	no	15 CPS	n.a.	1	35	AHS	CBZ
149	female	yes	2 CPS	n.a.	16	24	n.a.	PGB, LEV, OXC
150	male	yes	1 CPS	I A	8	42	AHS	VPA, LEV
151	male	yes	1 CPS	n.a.	12	n.a.	n.a.	LEV
152	male	yes	0-1 CPS	n.a.	30	n.a.	n.a.	OXC, ZON
153	female	yes	15 CPS	n.a.	16	n.a.	AHS	LTG, PGB
154	male	yes	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
155	female	yes	3 CPS	I B	38	41	n.a.	LEV, LTG
156	male	yes	3 CPS	I A	27	53	AHS	PHT
157	female	yes	2 CPS	I A	20	38	lesion associated	LEV, LTG
158	male	yes	0 CPS	n.a.	n.a.	n.a.	lesion associated	n.a.
159	male	yes	30 CPS	I B	27	36	lesion associated	ZON, PB, LEV, CBZ
160	male	yes	0-1 CPS	n.a.	9	n.a.	n.a.	TPM, LEV
161	female	yes	3 CPS	n.a.	43	45	n.a.	LEV, LTG
162	male	yes	4-6 CPS	IV B	4	30	lesion associated	CBZ, VPA
163	female	yes	1 CPS	n.a.	2	n.a.	n.a.	LEV
164	male	yes	4 CPS	I A	23	50	AHS	LEV, TPM, PGB
165	female	yes	30 CPS	I B	16	20	AHS	LEV, VPA, OXC
166	male	yes	2 CPS	I A	6	26	AHS	VPA, ZON
167	male	yes	1-2 CPS per week	I A	16	19	AHS	VPA
168	female	yes	< 1 CPS	n.a.	n.a.	n.a.	n.a.	LEV, CBZ
169	female	yes	< 1 CPS	n.a.	6	n.a.	n.a.	LEV, LTG
170	female	yes	0-1 CPS	n.a.	7	n.a.	n.a.	LTG, LEV
171	female	yes	< 1 CPS	n.a.	n.a.	n.a.	n.a.	LEV, LTG
172	male	yes	4 CPS	n.a.	5	35	AHS	LEV, CBZ
173	female	yes	3 CPS	I A	5	54	AHS	LTG, OXC
174	male	yes	< 1 CPS	n.a.	12	n.a.	n.a.	LEV, CBZ
175	male	yes	1 CPS	IV B	13	50	lesion associated	LTG
176	male	yes	4 CPS	I A	25	n.a.	n.a.	PHT
177	female	yes	< 1 CPS	n.a.	48	n.a.	n.a.	n.a.
178	female	yes	4 CPS	II B	4	36	AHS	CBZ, VPA, TPM
179	female	yes	< 1 CPS	n.a.	49	n.a.	n.a.	n.a.
180	female	yes	2 CPS	n.a.	6	56	AHS	LTG, CBZ
181	male	yes	3 CPS per day	I A	1	29	AHS	LEV, PGB, VPA
182	female	yes	2 CPS	I A	47	52	lesion asso-	LEV

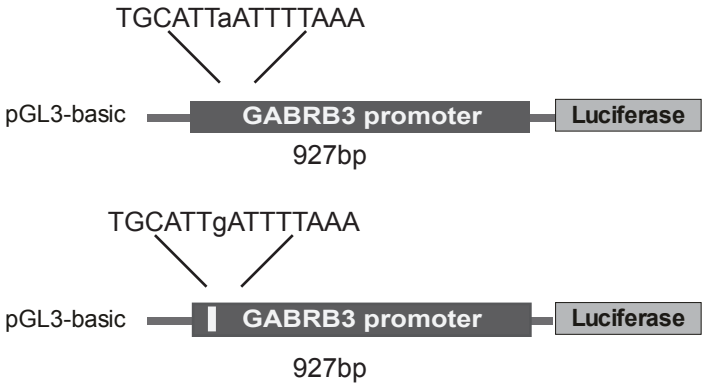
							ciated	
183	female	yes	1 CPS per week	IV B	33	37	AHS	LTG, LEV
184	male	yes	6 CPS	n.a.	3	n.a.	n.a.	LEV, LTG
185	male	yes	< 1 CPS	n.a.	6	n.a.	n.a.	LEV, LTG
186	male	yes	5-6 CPS per week	IV B	9	33	AHS	LTG, CBZ
187	male	yes	60 CPS	n.a.	4	n.a.	n.a.	LEV, PGB
188	male	yes	4 CPS	n.a.	30	n.a.	n.a.	LEV, PGB, PB
189	female	yes	6 CPS	I A	29	44	lesion asso-ciated	LTG, LEV
190	male	yes	0-1 CPS	IV B	34	65	lesion asso-ciated	CBZ, LEV
191	female	yes	0-1 CPS	n.a.	12	n.a.	n.a.	LEV, LTG, VPA
192	male	yes	< 1 CPS	n.a.	39	n.a.	lesion asso-ciated	LEV, LTG
193	female	yes	3 CPS	n.a.	3	n.a.	AHS	VPA, OXC
194	female	yes	3 CPS	n.a.	32	n.a.	n.a.	LEV, VPA, PRM
195	male	yes	1 CPS	I A	6	45	AHS	CBZ
196	male	yes	< 1 CPS in 2 years	n.a.	6	n.a.	n.a.	CBZ, LEV
197	male	yes	0-1 CPS	I A	28	35	lesion asso-ciated	OXC, LEV
198	female	yes	4 CPS	I A	13	28	AHS	LEV, CBZ
199	male	yes	n.a.	I B	20	21	lesion asso-ciated	n.a.
200	female	yes	7 CPS	I A	11	53	AHS	LEV, LTG
201	male	yes	1 CPS	I A	30	34	AHS	LEV, PGB
202	male	yes	n.a.	IV B	29	n.a.	n.a.	CBZ, LTG
203	male	yes	2 CPS per year	n.a.	2	n.a.	n.a.	LEV, CBZ, PB

Pernhorst et al., Supplemental Figure 1, Supplemental Digital Content 3

A



B



Luciferase reporter constructs including different promoter variants of candidate genes.
(A) We inserted a 827bp promoter fragment (black box) of the predicted promoter region upstream of the *ALDH5A1* gene upstream of the luciferase reporter gene. The short sequences above the fragments represent the different promoter variants (a lowercase c for the wildtype and a lowercase a for the SNP variant). **(B)** We cloned two different 927bp long fragments of the putative *GABRB3* promoter region in front of the luciferase reporter gene. The sequence variations were denoted by lowercase letters (a for the wildtype fragment and g for the SNP fragment).

Pernhorst et al., Supplemental Table 3, Supplemental Digital Content 4:

Genotype distributions of candidate SNPs

A post-hoc testing of the genotype distributions of the candidate SNPs stratified according to distinct individual/clinical characteristics. Variables are expressed in absolute numbers. A X^2-test was applied with a type I error rate of P = 0.05. Intriguingly, this co-variance analysis results in only depressive symptoms from the list of individual clinical parameters to correlate significantly with distinct genotypes of the genes under study (Post-operative outcome again according to Engel's classification).

A) Clinical data of mTLE patients: correlation to *ALDH5A1* genotypes.

	Genotypes				
Clinical characteristics	**AA**	**AC**	**CC**	**X^2**	**P**
Gender (Male / Female)	16/17	48/48	34/25	1.06	0.59
Depression (yes / no)	22/11	28/66	26/33	**14.1**	**0.001**
Number of seizures per month (0-10 / 11-20 / 21- more than 100)	28/0/4	66/12/14	43/5/9	5.27	0.26
Seizure-free post-operative (I A/ I B/ II B/ IV B)	9/3/0/12	44/2/4/33	27/2/2/19	6.62	0.35
Age at seizure onset in years (1-10 / 11-50)	16/14	57/36	26/31	3.54	0.16
Age at epilepsy surgery in years (1-10 / 11-64)	3/22	10/75	2/49	2.57	0.31
Pathology (AHS / Rasmussen's encephalitis / lesion associated)	23/0/4	69/1/17	37/1/11	1.32	0.9
Drug therapy (sodium channel blocker monotherapy / levetiracetam combinations / non-levetiracetam combinations)	5/15/10	21/32/37	14/22/20	2.3	0.68

B) Clinical data of mTLE patients: correlation to *GABRB3* genotypes.

	Genotypes				
Clinical characteristics	**AA**	**AG**	**GG**	**X^2**	**P**
Gender (Male / Female)	64/60	28/25	10/4	1.99	0.4
Depression (yes / no)	57/64	15/38	9/5	**8.08**	**0.017**
Number of Seizures per month (0-10 / 11-20 / 21- more than 100)	89/12/18	38/4/9	11/1/0	2.69	0.61
Seizure-free post-operative (I A/ I B/ II B/ IV B)	53/6/3/39	19/2/3/24	6/1/0/4	4.09	0.66
Age at seizure onset in years (1-10 / 11-50)	63/51	29/23	5/9	2.02	0.39
Age at epilepsy surgery in years (1-10 / 11-64)	10/95	5/43	0/11	1.21	0.64
Pathology (AHS / Rasmussen's encephalitis / lesion associated)	76/2/23	39/0/12	9/0/2	1.4	0.86
Drug therapy (sodium channel blocker monotherapy / levetiracetam combinations / non-levetiracetam combinations)	28/47/37	10/19/23	3/7/3	3.17	0.53

C) Clinical data of mTLE patients: correlation to gene expression of *ALDH5A1*

Clinical variables	**Mean**	**SEM**	**P**
Gender (Male / Female)	0.45 / 0.38	0.06 / 0.03	0.354
Depression (yes / no)	0.41 /0.41	0.09 / 0.03	0.969
Number of Seizures per month (0-10 / 11- more than 100)	0.41 / 0.45	0.03 / 0.09	0.607
Seizure-free post-operative (I A +II B/ I B + IV B)	0.43 / 0.40	0.05 / 0.04	0.734
Age at seizure onset in years (1-10 / 11-50)	0.41 / 0.41	0.05 / 0.04	0.998
Age at epilepsy surgery in years (1-10 / 11-64)	0.39 / 0.42	0.19 / 0.03	0.846
Pathology (AHS / Rasmussen's encephalitis + lesion associated)	0.43 / 0.28	0.04 / 0.04	0.114

D) Clinical data of mTLE patients: correlation to gene expression of *GABRB3*

Clinical variables	Mean	SEM	P
Gender (Male / Female)	0.16 / 0.16	0.01 / 0.01	0.644
Depression (yes / no)	0.14 /0.17	0.02 / 0.01	0.145
Number of Seizures per month (0-10 / 11- more than 100)	0.16 / 0.15	0.01 / 0.01	0.735
Seizure-free post-operative (I A +II B/ I B + IV B)	0.16 / 0.17	0.01 / 0.01	0.531
Age at seizure onset in years (1-10 / 11-50)	0.16 / 0.16	0.01 / 0.01	0.716
Age at epilepsy surgery in years (1-10 / 11-64)	0.16 / 0.16	0.02 / 0.01	0.891
Pathology (AHS / Rasmussen's encephalitis + lesion associated)	0.16 / 0.19	0.01 / 0.03	0.158

Pernhorst et al., Supplemental Table 4, Supplemental Digital Content 5:

Analysis of antiepileptic and antidepressant drug effects on gene expression of candidate genes and examination of antiepileptic drug treatment frequency in depressive patients.

A, **B**) The relative gene expression of candidate genes is given in patient groups stratified according to administration of distinct antiepileptic drugs with potential general effects on transcription, i.e. topiramate, valproic acid and levetiracetam. No significant differences in expression of ALDH5A1 or GABRB3 were present. Also antidepressants including Citalopram, Escitalopram, Haloperidol, Fluoxetine, Mirtazapine, Risperidone, Venlafaxine, Sertraline, Olanzapine and Amisulpride did not show effects on gene expression of both genes. Relative gene expression values are given as mean ± SEM. A two-tailed t-test was applied. **C**) Several antiepileptic compounds can result in mood disturbances. In order to analyze for respective effects of pharmacotherapy in the present patients, we analyzed potential overrepresentation of pharmacotreatment with topiramate, valproic acid and levetiracetam in patients with depression. However, depression was not significantly more frequent in patients treated with these antiepileptic compounds compared to individuals not receiving respective compounds.

A) Antiepileptic/antidepressant drug therapy: correlation to gene expression of *ALDH5A1*

Antiepileptic drug	Number of patients	Mean	SEM	P
Topiramat (yes / no)	3 / 34	0.30 / 0.42	0.04 / 0.03	0.288
Valproic acid (yes / no)	4 / 33	0.30 / 0.43	0.07 / 0.03	0.233
Levetiracetam (yes / no)	8 / 29	0.49 / 0.39	0.07 / 0.04	0.224
Antidepressants (yes / no)	6 / 31	0.39 / 0.42	0.08 / 0.04	0.755

B) Antiepileptic/antidepressant drug therapy: correlation to gene expression of *GABRB3*

Antiepileptic drug	Number of patients	Mean	SEM	P
Topiramat (yes / no)	5 / 51	0.18 / 0.16	0.02 / 0.01	0.497
Valproic acid (yes / no)	8 / 48	0.14 / 0.16	0.01 / 0.01	0.413
Levetiracetam (yes / no)	21 / 36	0.16 / 0.16	0.01 / 0.01	0.765
Antidepressants (yes / no)	7 / 49	0.14 / 0.16	0.02 / 0.01	0.345

C) Antiepileptic drug therapy: correlation to depression

		Depression +	Depression -	X^2	P
Number of patients		87	112		
Antiepileptic drug	Topiramat	10	9	0.26	0.61
	Valproic acid	12	29	2.36	0.124
	Levetiracetam	49	48	0.95	0.329

3.3 Summary

Human *GABRB3* and *ALDH5A1* genes encode for proteins both involved in GABA homeostasis. The *GABRB3* gene codes for a chloride channel subunit that serves as receptor for GABA. Downstream in the catabolism pathway of GABA, the *ALDH5A1* gene codes for succinic semialdehyde dehydrogenase (SSADH) that catalyzes an important step in the degradation of GABA. The inhibitory neurotransmitter GABA acts as essential regulator of excitability of neuronal networks (Mody and Pearce, 2004).
In this project, we focused on the question, how promoter variants impair stimulus-induced GABA homeostasis relevant gene expression. We concentrated on analyses of the promoter SNPs rs1883415 and rs4906902 in surgical brain tissue from patients with epilepsy. We further explored the potential influence of such genetic variants on the binding affinity of regulating TFs.
GABRB3 as well as *ALDH5A1* mRNA expression levels exhibit prominent differences correlating to the presence of respective SNP genotypes. Thus, *GABRB3* mRNA expression levels are significantly higher in brain tissue from TLE patients with the AA genotype compared to patients harboring the genotype GG of the SNP rs4906902. The same holds true for *ALDH5A1* mRNA expression. The group of TLE patients carrying the genotype AA of the SNP rs1883415 shows a significantly lower gene expression compared to the group of TLE patients homozygous for the C allele. Subsequently, we bioinformatically determined potential TFBSs at relevant SNP positions in order to uncover changes of TF binding affinity dependent on the respective SNP and their impact on the differential regulation of gene expression. The resulting data suggest that promoter SNPs affect the binding affinity of candidate TFs. In the promoter region of *GABRB3*, the activating TF Myocyte enhancer factor 2 (MEF-2) shows a higher binding affinity to the target sequence carrying the A allele than to the G allele sequence. In the case of *ALDH5A1*, we detected a potential binding site for the activating TF Early growth response 3 (Egr-3) with higher binding affinity to the C sequence variant

compared to the A sequence variant. In luciferase assays MEF-2 activated the A allele promoter variant significantly stronger than the G allele promoter variant. Concerning the bioinformatically predicted Egr-3 binding site in the *ALDH5A1* promoter, the exposure of Egr-3 to both promoter variants lead to a substantially increased promoter activation in the case of the C allele. Therefore, our results indicate that SNPs affect promoter activity by altering binding affinity of TFs.

Our data suggest that in human TLE, aberrant expression of genes coding for GABA-receptor subunits such as *GABRB3* as well as GABA-degrading enzymes such as *ALDH5A1* may result in an impairment of GABA metabolism and therefore disturb neurotransmission in affected brain tissue. Our data indicate the significance of promoter variants for the regulation of gene expression based on human brain tissue of patients with neurological brain disorders.

In addition to genes involved in GABA-mediated signaling, multiple studies revealed an evidence for promoter variants in genes known to be associated with hyperexcitability. Former studies point to a role of impaired serotonergic neurotransmission in facilitating seizure onset and inducing seizure severity (Lesch, 2001; Savic et al., 2004). Hence, the study of regulatory allelic variants affecting transcriptional changes in serotonin homeostasis-related genes, reveals an interesting aspect to investigate mechanisms in disorders characterized by episodic persistent onset of symptoms.

4 Rs6295 promoter variants of the serotonin type 1A receptor are differentially activated by c-Jun *in vitro* and correlate to transcript levels in human epileptic brain tissue

4.1 Introduction

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a main neurotransmitter in the CNS. In the case of epilepsy, serotonin was described to have an impact on seizure development, propagation and maintenance (Berumen et al., 2012). In addition, it was reported that an imbalance of serotonin levels is accompanied by both depressive symptoms and increased aggression (Siever, 2008; Kishi et al., 2009).

In general, released from serotonergic neurons in the raphe nucleus, serotonin activates G protein-coupled 5-HT receptors (5HT1ARs) located on the membrane of pyramidal and granular neurons (Wisden, 2010). 5HT1AR represents the most common subtype of seven classes of 5-HT receptors in the hippocampus. It plays a role in presynaptic as well as in postsynaptic signal transduction (Chaput et al., 1986; Hoyer et al., 1994, 2002). Animal studies using knockout mice lacking 5HT1AR revealed enhanced anxiety-related behavior (Gardier, 2009; Saxena and Chattopadhyay, 2011). Respective animals show a lower seizure threshold as well as an increased seizure activity, indicating 5HT1AR to be involved in neuronal excitability (Sarnyai et al., 2000). Likewise, selective inhibitors of serotonin uptake showed anticonvulsant effects both in experimental rat models (Pasini et al., 1992) and humans (Favale et al., 1995). Favale *et al.* suggest that the antiepileptic action of these inhibitors of serotonin uptake leads to anticonvulsant effects by potentiating serotonergic activity (Favale et al., 2003). Therefore, impairment of serotonergic homeostasis may play a role in the pathogenesis of neurological disorders including epilepsy.

The promoter region of the 5HT1AR encoding gene HTR1a comprises the SNP rs6295. This specific SNP has been associated with several CNS disorders including epilepsy, migraine and depression (Lemonde et al., 2003; Marziniak et al., 2007; Francois et al., 2008). Additionally, it has been further shown that the GG genotype of SNP rs6295 is related to the avoidance of physical activity during migraine attacks and that the SNP constitutes a risk factor for depression (Marziniak et al., 2007; Francois et al., 2008). The underlying mechanism was suggested to be an increase of 5-HT1A autoreceptor levels accompanied by reduced serotonin neurotransmission (Albert and Lemonde, 2004). Further, it was proposed that the SNP rs6295 modulates the response to antidepressant and antipsychotic treatment (Francois et al., 2008). However, the distinct effect of SNP variants on gene expression remained unclear. Based on human hippocampal tissue, we want to investigate the functional relevance of SNP rs6295 for *HTR1a* expression as well as for binding affinity of transcriptional regulators. Therefore, we performed comprehensive bioinformatic, molecular genetic and *in vitro* approaches.

The subsequent manuscript published in January 2013 in *Brain Research* is allowed by Elsevier Journals to be include in a thesis or dissertation with full acknowledgment of the final article.

My contribution to this work covers the preparation of 140 human hippocampal brain tissue samples derived from pharmacoresistant TLE patients, DNA and mRNA isolation, the genotyping of samples dependent on SNP rs6295 using TaqMan SNP Genotyping Assay and real-time RT-PCR experiments comparing sample groups according to their SNP rs6295 genotype. Additionally, I generated the reporter plasmid constructs for transient transfections in cell culture and subsequently performed luciferase assay experiments in order to clarify the *in silico* prediction of potential TFBSs as well as Chromatin immunoprecipitation assay (ChIP) assays to verify the binding of c-Jun to *HTR1a* promoter. These also included the bioinformatic and statistical analyses and the writing of the manuscript.

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Brain Research

Research Report

Rs6295 promoter variants of the serotonin type 1A receptor are differentially activated by c-Jun in vitro and correlate to transcript levels in human epileptic brain tissue

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ABSTRACT

Many brain disorders, including epilepsy, migraine and depression, manifest with episodic symptoms that may last for various time intervals. Transient alterations of neuronal function such as related to serotonin homeostasis generally underlie this phenomenon. Several nucleotide polymorphisms (SNPs) in gene promoters associated with these diseases have been described. For obvious reasons, their regulatory roles on gene expression particularly in human brain tissue remain largely enigmatic. The rs6295 G-/C-allelic variant is located in the promoter region of the human *HTR1a* gene, encoding the G-protein-coupled receptor for 5-hydroxytryptamine (5HT1AR). In addition to reported transcriptional repressor binding, our bioinformatic analyses predicted a reduced binding affinity of the transcription factor (TF) c-Jun for the G-allele. In vitro luciferase transfection assays revealed c-Jun to (a) activate the rs6295 C- significantly stronger than the G-allelic variant and (b) antagonize efficiently the repressive effect of Hes5 on the promoter. The G-allele of rs6295 is known to be associated with aspects of major depression and migraine. In order to address a potential role of rs6295 variants in human brain tissue, we have isolated DNA and mRNA from fresh frozen hippocampal tissue of pharmacoresistant temporal lobe epilepsy (TLE) patients ($n=140$) after epilepsy surgery for seizure control. We carried out SNP genotyping studies and mRNA analyses in order to determine *HTR1a* mRNA expression in human hippocampal samples stratified according to the rs6295 allelic

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variant. The mRNA expression of *HTR1a* was significantly more abundant in hippocampal mRNA of TLE patients homozygous for the rs6295 C-allele as compared to those with the GG-genotype. These data may point to a novel, i.e., rs6295 allelic variant and c-Jun dependent transcriptional 5HT1AR 'receptoropathy'.

1. Introduction

Impaired serotonin homeostasis is a frequent pathogenetic aspect in many neuropsychiatric disorders including depression, migraine and epilepsy (Trindade-Filho et al., 2008; Bahremand et al., 2011; Richerson and Buchanan, 2011; Theodore et al., 2012). The episodic, sustained onset of symptoms in these disorders suggests transient alterations of serotonin signaling due to factors such as transcriptional changes of relevant receptors (Lambru and Matharu, 2011; Richerson and Buchanan, 2011; Theodore et al., 2012). The serotonin-1A receptor (5HT1AR) is a central component in activity regulation of the serotonin system, and is expressed in many neuronal structures including the hippocampus where it serves as major postsynaptic receptor of neurons (Le Francois et al., 2008). Association studies suggest that genetic variation of the *HTR1a* gene influences the risk for a broad range of neuropsychiatric disorders (Lemonde et al., 2003; Marziniak et al., 2007; Le Francois et al., 2008). Lack of common missense variations in the *HTR1a* gene may suggest that regulatory sequence variations affecting gene expression of the *HTR1a* gene exert the functional effects.

Here, we have investigated the functional role of the single nucleotide polymorphism (SNP) rs6295 located in the promoter of *HTR1a* for the expression of the respective gene in human brain tissue. Rs6295 shows prominent associations in several neuropsychiatric disorders. In migraineurs, the rs6295 GG-genotype is associated with avoidance of physical activity during migraine attacks (Marziniak et al., 2007). The G-allele of rs6295 is linked to major depression, panic disorders and neuroticism (Lemonde et al., 2003; Le Francois et al., 2008). The G-allele of rs6295 is further related to reduced response to antidepressant or antipsychotic treatment (Le Francois et al., 2008). Rs6295 has been demonstrated as a functional promoter variant affecting binding sites for transcriptional repressors such as NUDR/Deaf1 and Hes5 (Albert et al., 2011). Yeast one-hybrid analyses suggested repression of the *HTR1a* promoter activity by NUDR and Hes5 preferentially at the rs6295 C-allele (Lemonde et al., 2003). These data implied that the rs6295 G-allele mediates a reduced repression of the *HTR1a* promoter, which leads to higher *HTR1a* mRNA levels in brain tissue and thereby increases individual risk of neuropsychiatric disorders.

However, SNPs can have ambiguous roles for transcription factor binding. We have therefore used bioinformatic promoter analysis in order to find transcriptional activator binding affected by the rs6295 variants. Based on respective results, we have analyzed a potential functional role of the transcriptional activator c-Jun on rs6295 in vitro. Given the association of rs6295 with episodic brain disorders and the in vitro evidence for an allele-specific regulation of the *HTR1a* promoter activity, we have subsequently explored whether the rs6295 alleles influence *HTR1a* mRNA expression in *human brain tissue*. Therefore, we used our unique access to fresh frozen hippocampal tissue of temporal lobe epilepsy (TLE) patients that underwent epilepsy surgery for seizure control. A previous association study did not support a risk-conferring effect of the *HTR1a* promoter SNP rs6295 in TLE (Stefulj et al., 2010). Due to the high number of neuropsychiatric co-morbidities in TLE, we further addressed potential clinico-genetic associations of rs6295 in TLE patients as addressed in a previous study in our present TLE patient series (Theodore et al., 2012).

2. Results

2.1. *Bioinformatic promoter analysis reveals a c-Jun binding site at rs6295 with allele specific binding affinities*

We have used bioinformatic promoter analysis in order to find transcriptional activator binding affected by the rs6295 variants. We predicted the position and conservation of a putative binding site for c-Jun using position-specific-scoring matrices in the *HTR1a* promoter region (Fig. 1A). The rs6295 C-variant exhibits a potential c-Jun binding motif with a higher 'motif similarity score', i.e., binding affinity compared to an 'ideal' binding motif, compared to the c-Jun binding motif in the G-variant (73% vs. 65%; Fig. 1A and B). Using chromatin immunoprecipitation (ChIP) assays, we observed substantial binding of c-Jun at the *HTR1a* promoter on protein level (Student's *t*-test: $^{**}p=0.0025$; $n=14$; Fig. 1C–D).

2.2. *c-Jun exposure results in stronger activation of the rs6295 C-allelic than G-allelic variant*

We next addressed the biological impact of this bioinformatic prediction by using luciferase reporter constructs including different promoter variants of *HTR1a* at the rs6295 site (Supplemental Fig. 1). In a first experiment, we addressed the basal *HTR1a* promoter activity for the both rs6295 variants. This activity was not significantly different for the rs6295 C-allelic versus the G-allelic variant (Student's *t*-test: $p=0.081$; $n=4$ each; Fig. 1E). Increased levels of c-Jun strongly activate both *HTR1a* promoter variants compared to respective unstimulated controls (C-allele: one-way ANOVA: $^{***}p<0.001$; G-allele: one-way ANOVA: $^{*}p<0.05$; $n=4$ each; 20 ng c-Jun; Fig. 2A). Our data furthermore demonstrate that the transcription factor c-Jun activates the rs6295 C-allelic variant significantly stronger than the rs6295 G-allele (one-way ANOVA: $^{*}p<0.05$; 20 ng c-Jun; Fig. 2A). Intriguingly, the expression levels of c-Jun in hippocampi of TLE patients included in the present series harboring the rs6295 C-allele are higher compared to c-Jun expression in individuals

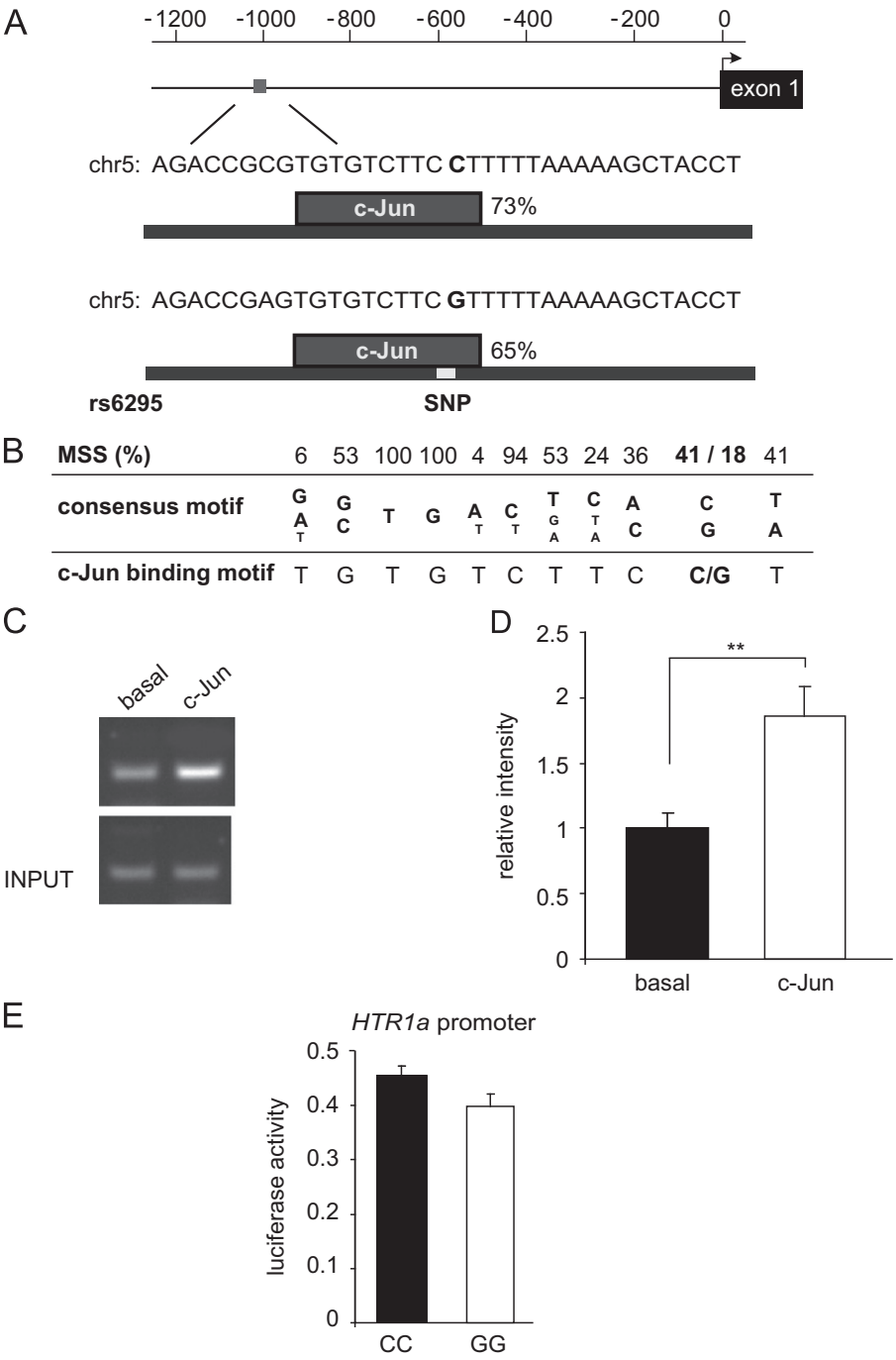

Fig. 1 – Bioinformatic analysis of TF-binding site modifications in the *HTR1a* promoter. (A) The promoter of the *HTR1a* gene comprises the rs6295 SNP 1019 bp upstream of transcriptional start 'ATG'. We identified a putative binding site for the activating transcription factor c-Jun in the C-sequence with a binding affinity of 73%. In the G-variant of the *HTR1a* promoter sequence, the predicted binding affinity of this TF-binding site is 65%. (B) The predicted c-Jun binding motif in the *HTR1a* promoter region is represented at each position with the corresponding matrix similarity score (MSS) as a percentage. In the consensus motif of c-Jun, the size of each nucleotide denotes the frequency of the nucleotide at this position. (C) A representative PCR analysis of ChIP products shows the binding of c-Jun to the *HTR1a* promoter. (D) Quantification of the ChIP experiment demonstrates a significant binding of c-Jun to the *HTR1a* promoter compared to the basal promoter (Student's *t*-test: $^{**}p=0.0025$; $n=14$). (E) The basal luciferase activity of the CC-(black bar) and GG-promoter fragments (white bar) of *HTR1a* did not differ significantly (Student's *t*-test: $p=0.081$; $n=4$ each).

homozygous for the rs6295 G-allele (Student's *t*-test: $^{*}p=0.039$; Fig. 4C). The previously reported transcriptional repressors NUDR and Hes5 were analyzed by adding 10 ng of each transcriptional repressor. Thereby, we observed a significant inhibition of *HTR1a* promoter activity by NUDR for both genotypes (C-allele: Student's *t*-test: $^{***}p=2\times10^{-8}$;

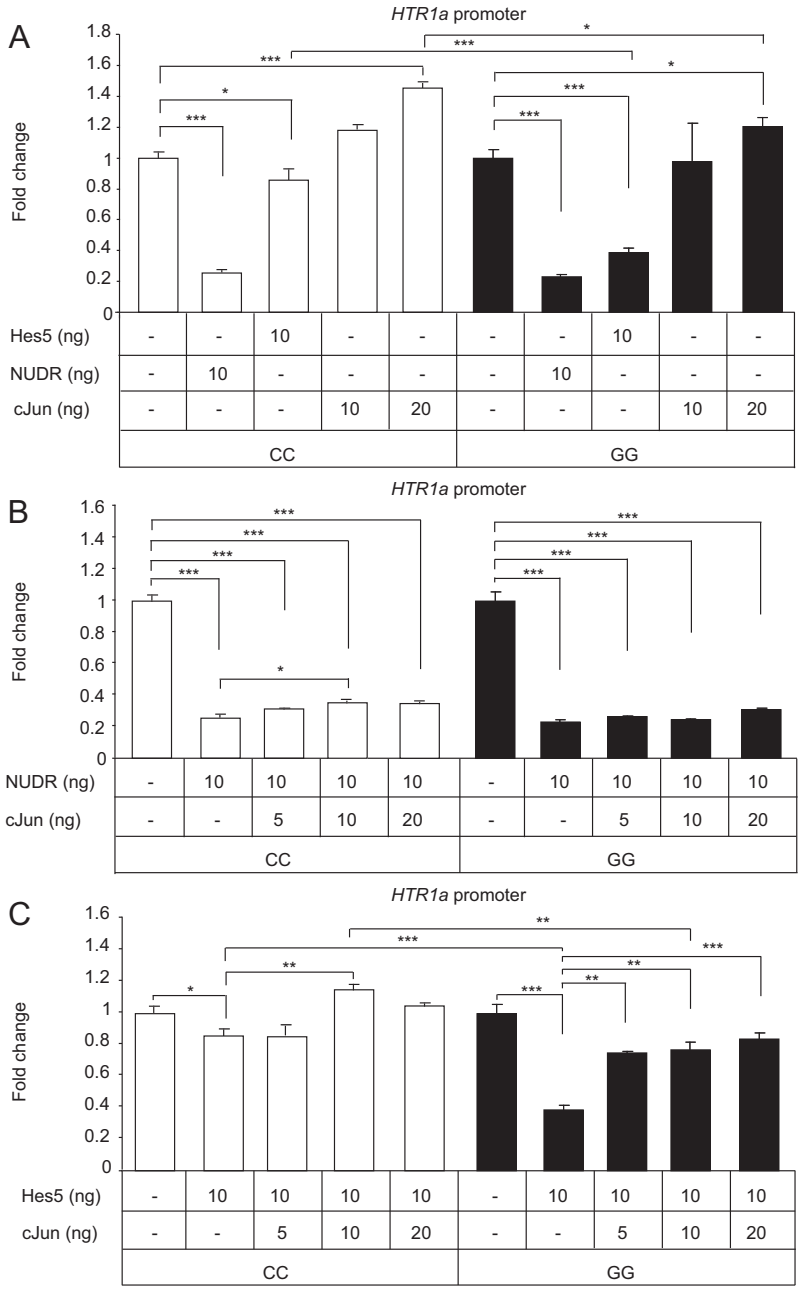

Fig. 2 – In vitro luciferase activity analysis of ***HTR1a*** promoter variants. **(A) Adding 10 ng of the transcriptional repressor NUDR leads to a significant inhibition of *HTR1a* promoter activity for both genotypes (C-allele: Student's *t*-test: $^{***}p = 2 \times 10^{-8}$; $n = 4$ each; white bars; G-allele: Student's *t*-test: $^{***}p = 3 \times 10^{-7}$; $n = 4$ each; black bars). The same holds true for the transcriptional repressor Hes5 (C-allele: Student's *t*-test: $^{*}p = 0.037$; $n = 4$ each; white bars; G-allele: Student's *t*-test: $^{***}p = 4 \times 10^{-5}$; $n = 4$ each; black bars). There is a stronger repressing effect of Hes5 on the promoter G-variant compared to the C-variant (one way ANOVA: $^{***}p < 0.001$; $n = 4$). Both promoter fragments homozygous for the G- and C-alleles are significantly activated after overexpression of c-Jun (20 ng) compared to basal states (C-allele: one-way ANOVA: $^{***}p < 0.001$; $n = 4$ each; white bars; G-allele: one-way ANOVA: $^{*}p < 0.05$; $n = 4$ each; black bars). Intriguingly, there is a substantially stronger activation of the promoter fragment carrying the rs6295 C-allele (white bar) compared to the G-allele (black bar) after exposure of both *HTR1a* promoters with 20 ng c-Jun (one-way ANOVA: $^{*}p < 0.05$; $n = 4$ each). (B) Exposure of only 10 ng NUDR and increasing concentrations of c-Jun in combination with 10 ng of the transcriptional repressor NUDR to both genotypes leads to a significant decrease in promoter activity compared to basal level (one-way ANOVA: $^{***}p < 0.001$; $n = 4$). After combined overexpression of c-Jun (10 ng) and NUDR (10 ng), the *HTR1a* promoter activity is significantly increased compared to overexpression of NUDR alone in the CC-genotype (one-way ANOVA: $^{*}p < 0.05$; $n = 4$ each; white bars). (C) For both the C- and the G-variant, the *HTR1a* promoter is significantly repressed after exposure to Hes5 alone compared to basal level (one-way ANOVA: $^{*}p < 0.05$; $^{***}p < 0.001$; $n = 4$ each). The *HTR1a* promoter activity is significantly increased when simultaneously adding c-Jun and Hes5 (10 ng each) for the C-variant and after exposure of combined Hes5 (10 ng) with increasing concentration of c-Jun for the G-variant compared to overexpression of Hes5 alone (one-way ANOVA: $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 4$ each). For the C-variant only the combined overexpression of c-Jun and Hes5 showed a reversible effect by adding c-Jun. The comparison of the *HTR1a* promoter activity after overexpression with Hes5 and c-Jun (10 ng each) showed a significantly stronger effect for the promoter fragment homozygous for the C-allele (one-way ANOVA: $^{**}p < 0.01$; $n = 4$ each).**

G-allele: Student's *t*-test: $^{***}p=3\times10^{-7}$; $n=4$ each; Fig. 2A). The same holds true for the transcriptional repressor Hes5 (C-allele: Student's *t*-test: $^{*}p=0.037$; G-allele: Student's *t*-test: $^{***}p=4\times10^{-5}$; $n=4$ each; Fig. 2A). To address the question whether the inhibitory effects of the transcriptional repressors NUDR and Hes5 are antagonized by the activating TF c-Jun, we performed in vitro luciferase assays where we exposed the respective promoter to combinations of either NUDR or Hes5 with c-Jun. After combined overexpression of c-Jun (10 ng) and NUDR (10 ng), the *HTR1a* promoter activity is significantly increased compared to overexpression of only NUDR (10 ng) in the CC-genotype, even though the absolute differences of promoter activation are not very large (one-way ANOVA: $^{*}p<0.05$; $n=4$ each; Fig. 2B). For the CC- as well as the GG-genotype the combined overexpression of c-Jun and Hes5 showed a significant repressor-antagonizing effect of c-Jun (one-way ANOVA: $^{**}p<0.01$; $n=4$ each; Fig. 2C). Interestingly, the comparison of the *HTR1a* promoter activity after overexpression with Hes5 and c-Jun (10 ng each) showed a significantly stronger effect for the promoter fragment homozygous for the rs6295 C-allele than for the G-allele (one-way ANOVA: $^{**}p<0.01$; $n=4$ each; Fig. 2C).

2.3. *Different HTR1a expression levels in human hippocampal mRNA of individuals homozygous for the rs6295 C- versus G-allele*

Human hippocampal biopsy samples derived from epilepsy surgery for seizure control of pharmacoresistant TLE patients represent a unique resource of human brain tissue for molecular analyses (Fig. 3A). In order to examine potentially differential allele-specific expression levels, we compared *HTR1a* mRNA expression in the brain tissue samples of TLE patients homozygous for the rs6295 C-allele (C/C; $n=23$) with transcript levels of patients homozygous for the G-allele (G/G; $n=20$). The relative gene expression of *HTR1a* was 2.2-fold significantly higher in hippocampi of TLE patients homozygous for the rs6295 C-allele compared to those homozygous for the G-allele (Student's *t*-test: $^{*}p=0.013$; Fig. 3B). Additionally, the TLE patients homozygous for the C-allele compared to patients harboring the rs6295 CG-genotype demonstrated a significantly higher mRNA expression level for *HTR1a* (CC vs. CG: one-way ANOVA; $^{*}p<0.05$; Supplemental Fig. 2). Comparing transcript levels of patients with the rs6295 CG-genotype to patients homozygous for G-allele revealed no significant difference of respective mRNA expression.

2.4. *Clinical parameters of TLE patients do not correlate with the allelic variants of rs6295 or HTR1a mRNA expression*

With respect to the reported associations of distinct rs6295 genotypes with several neuropsychiatric disorders, we carried out a comprehensive co-variance analysis of clinical, therapeutic and co-morbidity data of the patients included here (Supplemental Tables 1–4). These analyses did not result in significant associations of particular rs6295 genotypes or *HTR1a* mRNA expression with any of the individual parameters investigated. Therefore, the significant differences in *HTR1a* transcript levels in individuals homozygous for the C- versus G-alleles of rs6295 point to a substantial allele-specific effect on promoter activity in human brain tissue.

2.5. *Transcriptional repressors differentially binding rs6295 allelic variants are equally expressed in human hippocampal mRNA of individuals homozygous for the C- versus G-alleles*

It was previously demonstrated by others that the transcriptional repressors NUDR and Hes5 have a higher binding affinity to the rs6295 C-allele of the *HTR1a* promoter (Lemonde et al., 2003; Albert et al., 2011). Therefore, our finding of a higher *HTR1a* mRNA expression level in hippocampi of TLE patients carrying the CC- genotype was unexpected and seemed potentially contradictory to our initial hypothesis. In a next step, we analyzed the mRNA expression of respective transcriptional repressors in human hippocampal mRNA of our TLE patients. We did, however, not observe differential expression of the transcriptional repressors NUDR and Hes5 in individuals homozygous for the different rs6295 alleles (Fig. 4A and B), which would have provided a potential explanation for our initial observation of differential *HTR1a* mRNA levels in individuals homozygous for the C- versus G-alleles of rs6295. Unfortunately, control human hippocampal tissue was not available for further mRNA expression analyses.

3. Discussion

Our present results suggest that the transcription factor c-Jun has significantly different activating effects on rs6295 SNP promoter *HTR1a* variants, i.e., c-Jun binds in vivo to the *HTR1a* promoter and more strongly activates the C-variant (Fig. 1A and B). Furthermore, we confirmed the previously reported repressing effects of NUDR and Hes5 on the promoter (Lemonde et al., 2003; Albert et al., 2011). Our own bioinformatic TF binding site analyses at rs6295 for NUDR (C-allele: 61% and G-allele: 60%) and Hes5 (C-allele: 56.6% and G-allele: 46.6%) as well as the luciferase assays suggest, however, a stronger repressive effect for Hes5 for the rs6295 SNP G-variant (Fig. 2C). Our data demonstrate that rs6295 is located in an effective binding site for transcriptional repressors as well as activators in the *HTR1a* promoter. In this context, rs6295 has significant effects on the promoter activity modulation by respective transcriptional repressors and activators. How do these effects interfere? In the case of NUDR, although there is a statistically significant antagonistic effect on repression by c-Jun on the C-variant promoter, the effect size appears biologically not prominent (Fig. 2B). Intriguingly, c-Jun has the potency of strongly antagonizing the inhibitory effect of Hes5 in both promoter variants (Fig. 2C). However, there is significantly stronger *HTR1a* promoter activity in the case of exposure to Hes5 and c-Jun for the rs6295 C- instead of G-variant. This result may be due to additive functions by (a) the stronger activating effect of c-Jun and (b) the attenuated repressive effect of Hes5 on the C-variant. Whereas for NUDR, its repressive effect on the promoter is strongly evident independent of the presence of c-Jun in both SNP variants, Hes5 and c-Jun in similar

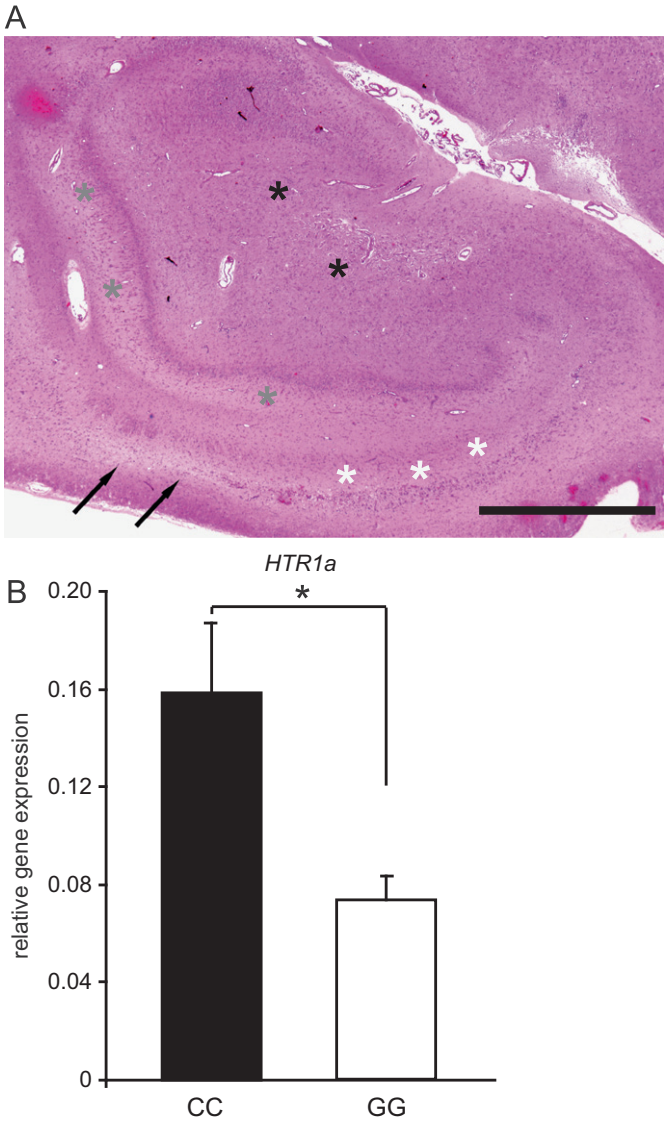

Fig. 3 – *HTR1a* mRNA expression in human hippocampal tissue. (A) A representative section of a bioptic human hippocampal specimen of a pharmacoresistant TLE patient after selective amygdalahippocampectomy shows the characteristics of hippocampal sclerosis (HS) with reduced neuronal densities particularly in CA1 and CA3/4 and concomitant astrogliosis (hematoxylin & eosin staining; grey asterisk—dentate gyrus (DG) granule cell layer; black asterisk—CA4; white asterisk—CA2; black arrows—CA1; bar graph—2.0 mm), i.e., containing major CNS cell populations. (B) The relative mRNA expression of *HTR1a* is significantly increased in the group of TLE patients homozygous for the rs6295 C-allele ($n=23$; 0.16 ± 0.03; black bar) compared to the group carrying the GG genotype ($n=20$; 0.07 ± 0.01; Student's *t*-test: $^{*}p=0.013$; white bar).

concentrations virtually neutralize each other's effects in the *HTR1a* promoter (Fig. 2C).

Certainly, it can only be speculated on respective effects and interplay of the repressors and c-Jun in vivo in depression and migraine, since respective tissue samples for quantification of transcriptional repressors and activators as well as promoter binding studies are not available. Some of these limitations can be overcome in hippocampal tissue of TLE patients. More abundant *HTR1a* mRNA was present in individuals with the rs6295 CC-genotype compared to patients with the GG-genotype. However, for transcript analyses in true human control hippocampi the obvious lack of respective tissue samples remains an obstacle. Furthermore, it cannot entirely be resolved how acute and dynamic expression alterations of the transcriptional repressors and c-Jun dependent on seizure activity in patients affect expression levels of *HTR1a*.

Intriguingly, c-Jun expression levels are higher in hippocampal mRNA of TLE patients with the rs6295 CC-genotype compared to those with the GG-genotype. The cause of the latter observation is not clear. However both factors, i.e., stronger *HTR1a* promoter activation of the rs6295 C-allele and higher expression levels of c-Jun in hippocampal tissue of respective individuals should be independent factors adding up in a functional fashion with respect to expression of *HTR1a*. Although in luciferase assays, there is no different

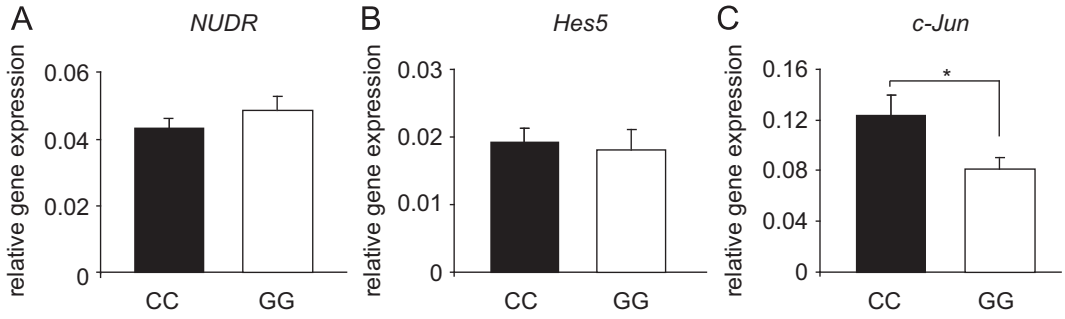

Fig. 4 – The mRNA expression of transcriptional repressors and activating TFs. (A) The relative mRNA expression for the transcriptional repressor NUDR in rs6295 CC-genotype patients ($n=17$; 0.043 ± 0.003; black bar) differs not significantly compared to GG-genotypes patients ($n=16$; 0.048 ± 0.004; Student's *t*-test: $p=0.285$; white bar). (B) The same holds true for the transcriptional repressor Hes5 in patients with the CC-genotype ($n=17$; 0.019 ± 0.002; black bar) compared to GG-genotype TLE individuals ($n=16$; 0.018 ± 0.003; Student's *t*-test: $p=0.768$; white bar). (C) c-Jun expression in patients with the rs6295 CC-genotype ($n=17$; 0.123 ± 0.016; black bar) is significantly higher compared to patients homozygous for the rs6295 G allele ($n=16$; 0.082 ± 0.009; Student's *t*-test: $^{*}p=0.039$; white bar).

basal activity of the rs6295 CC- compared GG-promoter variants, the situation with respect to dynamics of the transcription factor environment may be even more complex and different in human hippocampal tissue. c-Jun expression and the rs6295 genotypes in human hippcampal tissue in this context did not co-vary with respect to *HTR1a* transcript levels (data not shown). In status epilepticus animal models, increased hippocampal expression of c-Jun has been reported (Han et al., 2009). The hippocampal expression levels of c-Jun after chronic spontaneous seizure activity is difficult to determine. In the chronic stage after status epilepticus, animals are desynchronized with respect to seizure activity and c-Jun is extremely variable in mRNA expression after episodic insults such as spontaneous seizures. However, we speculate that there is increase of c-Jun as an immediate early gene after seizure activity.

Our data suggest that in addition to previously reported transcriptional repressors, the *activating* TF c-Jun as a prominent immediate early gene has a substantial impact on mRNA expression levels of *HTR1a* in human epileptic brain tissue. However, our findings clearly relate to the situation of TLE. For TLE, the present data suggest a scenario in which putatively transiently increased expression levels of c-Jun, e.g. in the context of enhanced neuronal activity levels (Sheng and Greenberg, 1990) substantially alter the expression of *HTR1a* in individuals harboring the C-allele of rs6295 compared to basal states. This effect presumably leads to impaired serotonin homeostasis. Our data in human TLE does not allow direct extrapolation onto other neuropsychiatric disorders such as depression and migraine since the expression levels of relevant transcriptional repressors and activators may be entirely different in these disease contexts. However, our data suggest that the modulatory effect of transcription factor binding at rs6295 is not restricted to repressor proteins.

In TLE, however, our data propose substantial effects of the rs6295 genotype mediated by c-Jun on *HTR1a* expression. Whether this data points to a novel form of a transcriptional acquired 5HT1AR 'receptoropathy' needs to be clarified by future, e.g., electrophysiological and animal model functional approaches.

4. Experimental procedure

4.1. *Patient criteria and surgical specimens*

We included hippocampal biopsy specimens of 140 pharmacoresistant TLE patients. The ascertainment scheme and clinical characteristics have been described in detail previously (Kral et al., 2002; Pernhorst et al., 2011) (Fig. 3A). Informed written approval was obtained from all patients and procedures were in accordance with the Declaration of Helsinki and approved by the local ethics committee. Depressive symptoms of patients were assessed according to Beck Depression Inventory (BDI; threshold BDI $\geq$12) (Beck et al., 1961). Anxiety and aggression scores were determined by a standardized questionnaire (Helmstaedter, 2001). Epilepsy-free controls for association studies belong to the cardiovascular longitudinal Heinz-Nixdorf-Recall study (HNR cohort; Schmermund et al., 2002).

4.2. *Sample preparation*

Processing of tissue, DNA and mRNA isolation, cDNA preparation and real time RT-PCR were carried out as described before (Pernhorst et al., 2011).

4.3. *SNP Genotyping analysis and real time RT-PCR*

Genotyping of SNP rs6295 was performed using a TaqMan SNP Genotyping Assay (HTR1a: C__11904666_10; Applied Biosystems, Foster City, CA, USA). The assignment of the alleles of the SNP rs6295 refer to the forward genome strand orientation relative to the NCBI reference genome build 36. Analysis of *HTR1a* mRNA expression was carried out as described before in detail (Pernhorst et al., 2011) (TaqMan Gene Expression Assay (HTR1a: Hs00265014_s1; Applied

Biosystems, Foster City, CA, USA). ß-actin was used as endogenous reference gene for normalization of the analyzed mRNAs.

4.4. Sample preparation and microarray analysis

Total RNA for gene expression microarray analysis was isolated from human hippocampal tissue samples using AllPrep DNA/RNA Mini Kit (Qiagen, Hilden) according manufacturer's protocol. In order to synthesize cDNA from total RNA and in vitro transcription to biotin-labeled cRNA, Illumina TotalPrep-96 RNA Amplification Kit (Life Technologies Corporation, Darmstadt) was used according to the manufacturer's protocol. For analysis of c-Jun mRNA expression, cRNA was then hybridized on HumanHT-12 v3 Expression BeadChips using lllumina Direct Hybridization Assay Kit (Life Technologies Corporation, Darmstadt, Germany). The Illumina BeadArray Reader was applied for scanning and resulted data was analyzed using Illumina's GenomeStudio Gene Expression Module. Gene expression data were normalized using the Illumina BeadStudio software suite by means of quantile normalization with background subtraction.

4.5. Reporter plasmids

The HTR1a-luciferase reporter plasmid was constructed by cloning a 1102bp fragment of the human *HTR1a* promoter harboring the SNP rs6295 that was amplified by PCR using human genomic DNA as template (Fwd-primer 5′-CTC GAG TGT TGT TGT CGT CGT TGT TC-3′, Rev-primer 5′-AGA TCT GGA AGG GGG AGG GAA GA-3′) and digested with *XhoI* and *BglII* into the luciferase reporter construct, pGL-3 basic (Promega Biotech, Madison, WI) (Supplemental Fig. 1).

4.6. Transient transfections

NG108-15 neuroblastoma cells were plated at a density of 80% confluency in 48-well plates and grown in 0.5 ml of DMEM supplemented with 10% v/v fetal calf serum, 5% Pen Strep and 5% HAT. Transfection of the cells was carried out using lipofectamine (Invitrogen, Darmstadt, Germany) following the manufacturer's protocol. For each well of the 48-well tissue culture plate, 50 ng luciferase reporter plasmid, 12.5 ng pRL-TK and 0.5 µl lipofectamine were mixed with 12.5 µl medium. Different amounts of a previously described vector containing a c-Jun expression sequence were used in respective experimental assays (Leppa et al., 1998). 10 ng of each repressing overexpression plasmid Hes5 (Nakashima et al., 2001) and NUDR (Michelson et al., 1999) were used. All mixtures were incubated for 20 min at room temperature and then added to the appropriate wells. Cells were grown in culture medium for 6–12 h at 37 °C and 5% CO_2. Thereafter, the medium was replaced by fresh medium and the cells were used for experiments 48 h after transfection.

4.7. Luciferase assays

Renilla luciferase was used to normalize the transfection efficiency data, and a Dual Luciferase Reporter Assay System was used according to the manufacturer's specifications (Promega, Biotech, Madison, WI). Renilla and firefly luciferase activities were determined using the Glomax Luminometer (Promega, Biotech, Madison, WI), counting each sample four times. The results are given as Firefly/Renilla relative light units if not indicated otherwise.

4.8. Chromatin immunoprecipitation (ChIP) assays

NG108-15 neuroblastoma cells (6-wells; 80% confluency) were transfected with pCMV-cJun or the empty pCMV vector using lipofectamine (Invitrogen, Darmstadt, Germany). The cells were crosslinked with 1% formaldehyde for 10 min at 37 °C 48 h after transfection followed by two washing steps in ice-cold PBS containing protease inhibitors (PIs; Roche Applied Science, Indianapolis, IN). The cells were collected, centrifuged and lysed in SDS lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris, pH 8.1 with PIs). An incubation step for 10 min on ice was attached. The ChIP sample processing has been described in detail elsewhere (van Loo et al., 2012). After phenol/chloroform extraction, the resulted DNA was analyzed by PCR using primers detecting the *HTR1a* promoter region: HTR1a_c-Jun_CHIP_FW: 5′-TGT CGT CGT TGT TCG TTT GT-3′; HTR1a_c-Jun_CHIP_REV: 5′-GGT GAA CAG TCC TGG GTC AG-3′; HTR1a_control_CHIP_FW: 5′-CCA GGT GCT GAA CCC AGT TT-3′; HTR1a_control_CHIP_REV: 5′–CAT GGC CTT TTG CAC TTC TT-3′. The following PCR was performed using following conditions: 94 °C for 3 min, then 35 cycles of 45 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, followed by a final extension step at 72 °C for 10 min. The PCR products were analyzed on 1.5% agarose gel and quantified using AIDA software.

4.9. Bioinformatic analyses

For identification of the potential promoter region upstream of the *HTR1a* gene, the web tools and software tools CpGPlot (Rice et al., 2000), Promoter2.0 (Knudsen, 1999), COMET (Frith et al., 2002) and Eponine (Down and Hubbard, 2002) were applied. To identify the position and conservation of potential transcription factor binding sites, PoSSuMsearch (Beckstette et al., 2006) using position-specific-scoring matrices (PSSMs) from the TRANSFAC database was applied to the potential promoter region. For filtering the potential transcription factor binding sites a motif similarity score (MSS) of 80% was applied.

4.10. Statistical analysis

A two-sided type I error rate of $P=0.05$ was chosen for the analyses. No correction for multiple testing was performed with respect to the exploratory design and modest statistical power of this study. Student's *t*-tests were used as indicated to evaluate the statistical significance of the normally distributed data sets of real time RT-PCR and ChIP analyses. One-way ANOVA followed by Bonferroni's multiple comparison tests were used to examine the statistical significance of the normally distributed luciferase assay results.

Acknowledgment

The mammalian expression vector c-Jun was kindly provided by Dirk Bohrmann (Rochester). We would also like to thank Dr. Tetsuya Taga (Tokyo, Japan) for providing the pEF-Bos-FlagHes5 expression vector and Dr. Jodi Huggenvik (Illinois, USA) for providing the h6NUDR expression vector. Our work is supported by Deutsche Forschungsgemeinschaft (KForG "Innate Immunity" TP2, AJB; Emmy Noether program: SS; SFB-645: SS), Bundesministerium für Bildung und Forschung (NGFNplus EMINet; AJB, SS, TS; Unabhängige Forschergruppen in den Neurowissenschaften, SS), EuroEPINOMICS CRP EpiGEN of the European Science Foundation (AJB), DFG Sa434/5-1 to TS, Else Kröner-Fresenius-Stiftung (AJB), German Israeli Foundation (AJB) & the BONFOR program of the University of Bonn Medical Center (AJB, SS).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2012.12.045.

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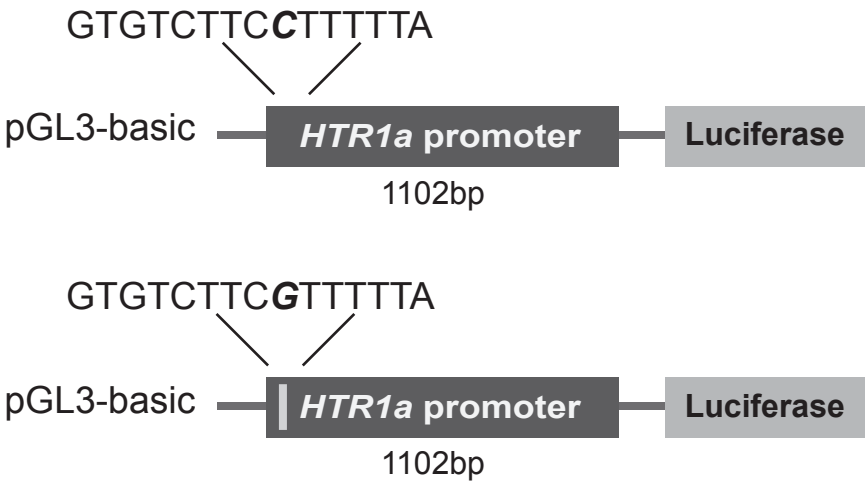
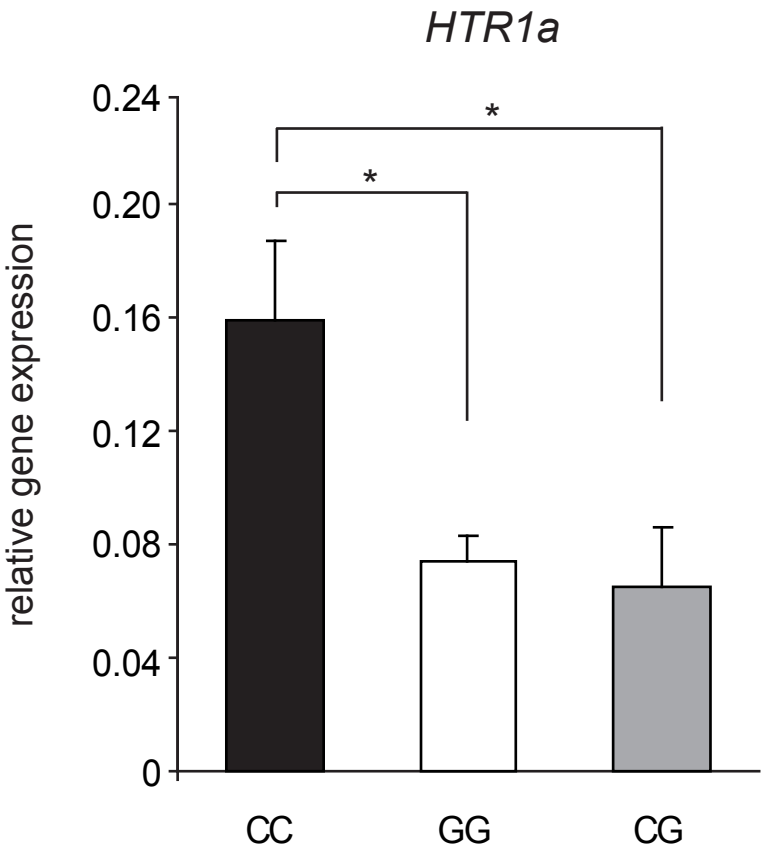
Pernhorst *et al.*, Supplemental Figure 1



Luciferase reporter construct including different promoter variants of *HTR1a* at the rs6295 site. We inserted a 1102bp promoter fragment (black box) of the predicted promoter region upstream of the *HTR1a* gene to control a luciferase reporter gene. The short sequences above the fragments represent the different promoter variants (a bold C for the major and a bold G for the minor rs6295 variant).

Pernhorst *et al.*, Supplemental Figure 2



The relative mRNA expression of *HTR1a* is significantly more abundant in the group of TLE patients homozygous for the rs6295 C-allele (n=23; 0.159 ± 0.03; black bar) compared to the group carrying the CG genotype (n=38; 0.065 ± 0.02; grey bar; one-way ANOVA with Bonferroni‘s multiple comparison tests: *p<0.05). Compared to the group of TLE patients homozygous for the rs6295 G-allele (n=20; 0.074 ± 0.01; white bar), the group of TLE patients carrying the CC-genotype showed a significant increase in relative mRNA expression of *HTR1a* (n=20; 0.07 ± 0.01; white bar; one-way ANOVA with Bonferroni‘s multiple comparison tests: *p<0.05). *HTR1a* expression in patients with the rs6295 GG-genotype differs not significantly compared to patients harbouring the rs6295 CG genotype.

Pernhorst *et al.*, Supplemental Table 1:

Summary of clinical patient parameters.

Table describing the clinical parameters of the TLE patients group including the number of all TLE patients, the number of blood samples, the number of brain samples, the percentage of gender, the percentage of pathology, mean of age at seizure onset in years, mean of age at epilepsy surgery in years and mean of BDI. Absolute number of patients with available clinical data is indicated in brackets. Biopsy specimens were neuropathologically analyzed according to standard procedures and hippocampi stratified according to the pathlogical pattern of the patient into the two following groups: AHS (Ammon's horn sclerosis) and lesion associated (ganglioglioma, cavernoma, dysembryoplastic neuroepithelial tumor). Notably, also hippocampi of patients with lesion associated TLE show substantial reactive astrogliosis in the hippocampal formation.

Number of patients	181
Number of blood samples	41
Number of brain samples	140
Gender (male / female) [181]	48.62 / 51.38
Pathology (AHS / lesion associated) [160]	77.71 / 22.29
Age of seizure onset [172]	12.45 ± 0.87
Age at epilepsy surgery [159]	32.45 ± 1.16
BDI [56]	32.45 ± 1.16

Pernhorst *et al.*, Supplemental Table 2

A) Genotype and allele frequency of the rs6295 SNP in the TLE group and of rs6449693 in the control group. Both the genotype and allele frequencies in the TLE group according to rs6295 and the genotype and allele frequencies in the control group according to rs6449693 are presented. Relative as well as absolute (in italics) genotype and allele frequencies are given. Results of X^2-test and two-tailed P value are shown. The genotype distribution in the control was not significantly different from those expected by Hardy-Weinberg equilibrium.

				Genotype frequency					Allele frequency			
SNP	**Gene**	**Group**	**n**	**CC**	**CG**	**GG**	**X^2**	**P**	**Allele C**	**Allele G**	**X^2**	**P**
rs6295	HTR1a	TLE	140	0.25 (*35*)	0.49 (*68*)	0.26 (*37*)	0.27	0.875	0.49 (*138*)	0.51 (*142*)	0.036	0.85
				AA	**AG**	**GG**			**Allele A**	**Allele G**		
rs6449693	HTR1a	Control*	2242	0.24 (*550*)	0.51 (*1136*)	0.25 (*556*)			0.5 (*2236*)	0.5 (*2248*)		

*Control according to HNR cohort

B) Genotype and allele frequency of the rs6295 SNP in patients suffering from TLE "with depression" versus those "without depression" groups. Additional blood DNA was obtained from TLE patients with depression (n=56), who have not yet undergone epilepsy surgery. Data is presented parallel to A). However, we do not observe significant differences in the allele or genotype frequency distributions between both groups (X^2-test and two-tailed P value).

					Genotype frequency					Allele frequency			
SNP	**Gene**	**Group**	**Depression**	**n**	**CC**	**CG**	**GG**	**X^2**	**P**	**Allele C**	**Allele G**	**X^2**	**P**
rs6295	HTR1a	TLE	**+**	70	0.3 (*21*)	0.471 (*33*)	0.229 (*16*)	0.79	0.67	0.536 (*75*)	0.464 (*65*)	0.836	0.36
		TLE	**-**	108	0.25 (*27*)	0.472 (*51*)	0.278 (*30*)			0.486 (*105*)	0.514 (*111*)		

Pernhorst *et al.*, Supplemental Table 3:

Genotype distributions of candidate SNP

The genotype distributions of the candidate SNP stratified according to distinct clinical characteristics. Variables are expressed in absolute numbers (X^2-test and two-tailed P value). Intriguingly, this co-variance analysis results in no significant correlation of individual clinical parameters with distinct genotypes of *HTR1a*.

A) Clinical data of mTLE patient: correlation to *HTR1a* genotypes.

Genotype characteristics	CC	CG	GG	X^2	P
Gender (Male / Female)	25/25	41/43	27/20	0.95	0.62
Number of Seizures per month (0-10 / 11-20 / 21- more than 100)	34/6/6	64/7/10	33/3/10	3,24	0.52
Age at seizure onset in years (1-10 / 11-50)	27/18	36/45	29/17	5.12	0.77
Age at epilepsy surgery in years (1-10 / 11-64)	4/39	7/68	2/39	0.8	0.67
Pathology (AHS / Rasmussen's encephalitis / lesion associated)	35/0/11	56/1/14	31/0/10	1.62	0.91
Drug therapy (sodium channel blocker monotherapy / levetiracetam combinations / non-levetiracetam combinations)	11/14/20	18/34/27	12/17/15	2.23	0.69

B) Clinical data of mTLE patients: correlation to gene expression of *HTR1a*.

Clinical variables	Mean	SEM	P
Gender (Male / Female)	0.12 / 0.12	0.03 / 0.02	0.911
Number of Seizures per month (0-10 / 11- more than 100)	0.11 / 0.13	0.02 / 0.04	0.618
Age at seizure onset in years (1-10 / 11-50)	0.13 / 0.11	0.02 / 0.02	0.592
Age at epilepsy surgery in years (1-10 / 11-64)	0.313/ 0.12	0.03 / 0.01	0.902
Pathology (AHS / Rasmussen's encephalitis + lesion associated)	0.12 / 0.13	0.02 / 0.03	0.859

Pernhorst *et al.*, Supplemental Table 4:

Genotype distributions of candidate SNP with response to antipsychotic treatment.
The genotype and allele distributions of the candidate SNP stratified with response to antipsychotic treatment. Variables are expressed in absolute numbers (X^2-test and two-tailed P value).

A) Association of the *HTR1a* promoter SNP rs6295 with response to antipsychotic treatment.

	Genotype frequency					Allele frequency			
Group	**CC**	**CG**	**GG**	**X^2**	**P**	**Allele C**	**Allele G**	**X^2**	**P**
antipsychotic treatment	0.17 (3)	0.53 (9)	0.29 (5)	0.92	0.63	0.44 (15)	0.56 (19)	0.62	0.43
no antipsychotic treatment	0.29 (46)	0.45 (73)	0.26 (42)			0.51 (165)	0.49 (157)		

B) Association of the *HTR1a* promoter SNP rs6295 with response to antipsychotic treatment in depressive patients.

	Genotype frequency					Allele frequency			
Group	**CC**	**CG**	**GG**	**X^2**	**P**	**Allele C**	**Allele G**	**X^2**	**P**
antipsychotic treatment	0.2 (2)	0.6 (6)	0.2 (2)	1.11	0.57	0.14 (10)	0.15 (10)	0.08	0.78
no antipsychotic treatment	0.32 (19)	0.42 (25)	0.25 (15)			0.86 (63)	0.85 (55)		

C) Association of the *HTR1a* promoter SNP rs6295 genotype with response to BDI level.

Rs6295 genotype	Mean of BDI	SEM	P
CC / GG	14.93 / 16.47	2.18 / 2.01	0.607

D) Correlation of the *HTR1a* gene expression to antiepileptic drug treatment using Bonferroni's Multiple Comparison Test.

	Mean difference	t	P value
Drug group 1 (sodium-channel blockers monotherapy) vs. drug group 2 (LEV-combinations)	0.09172	0.08194	P > 0.05
Drug group 1 (sodium-channel blockers monotherapy vs. drug group 3 (non-LEV combinations)	-1.354	1.347	P > 0.05
Drug group 2 (LEV-combinations) vs. drug group 3 (non-LEV combinations)	-1.446	1.354	P > 0.05

E) Correlation of the *c-Jun* gene expression to antiepileptic drug treatment using Bonferroni's Multiple Comparison Test.

	Mean difference	t	P value
Drug group 1 (sodium-channel blockers monotherapy) vs. drug group 2 (LEV-combinations)	480.3	1.69	P > 0.05
Drug group 1 (sodium-channel blockers monotherapy vs. drug group 3 (non-LEV combinations)	230.1	1.112	P > 0.05
Drug group 2 (LEV-combinations) vs. drug group 3 (non-LEV combinations)	-250.3	0.9077	P > 0.05

4.3 Summary

Not only epilepsies but many neuropsychiatric disorders manifest with episodic onset of symptoms. In respective disorders, large-scale genetic analyses have a characterized variety of allelic variants, often SNPs, that are non-coding. Many of them are located in putative gene promoter regions. These aspects hold particularly true for depression and migraine. For obvious reasons, brain tissue of respective patients is generally not available. However, native human brain tissue would allow intriguing insights, i.e. transcriptional analyses of corresponding genes in order to determine, whether distinct SNPs have promoter relevant activity. Biopsy brain tissue samples of pharmacoresistant temporal lobe epilepsy patients that undergo epilepsy surgery for seizure control represent a unique option in order to overcome these obstacles and stratify patients according to variants of the putative promoter SNPs for corresponding expression analysis of respective genes in human brain tissue.

By concentrating on a prominent SNP (rs6295) that has been associated with several neuropsychiatric disorders including migraine, we here followed this approach. Analysis of the SNP rs6295 and *HTR1a* expression in human TLE tissue occurred reasonable to us also in an epileptological context. The serotonin signaling system has been claimed to be relevant for focal epilepsies (Theodore et al., 2012). Furthermore rs6295 had been described as functional binding site for transcriptional repressors (Lemonde et al., 2003; Albert et al., 2011). Here, we have further explored, whether the respective promoter motif may in addition be a binding site for activating transcription factors. This may provide substantial more complex regulative potential of this prominent promoter site. In the present study we demonstrated a putative binding site for the transcription factor c-Jun at the position of the SNP rs6295 in the *HTR1a* promoter with allele-specific binding affinity using bioinformatic approaches. The allele-variants of SNP rs6295 affect the differential binding of c-Jun to the promoter region. As previously predicted, the C variant of the SNP rs6295 results in higher promoter activity com-

pared to the G variant after exposure with the activating TF c-Jun using *in vitro* reporter assays. The relevance of the SNP rs6295 on *HTR1a* expression levels is given by significant differences of mRNA expression in hippocampal tissue in patient groups homozygous for C and G alleles, respectively. In contrast to previous findings which demonstrate binding of transcriptional repressors NUDR and Hes5 to the SNP rs6295 C allele (Lemonde et al., 2003; Albert et al., 2011), we found an enhanced *HTR1a* expression in patients harboring the rs6295 genotype CC. In human hippocampal tissue originating from epilepsy surgery, the analysis of mRNA expression levels reveals no differences of transcriptional repressors NUDR and Hes5 in individuals homozygous for the SNP rs6295 alleles. Furthermore, the increased *HTR1a* expression correlates to the repressor-antagonizing effect of the activating factor c-Jun in patients homozygous for C allele.

In summary, this study indicates c-Jun as immediate early gene to have a modulatory effect on *HTR1a* expression. With respect to an increase in neuronal activity in surgical brain tissue of TLE patients under study, our data suggest a transient rise of c-Jun expression. This leads to alterations in *HTR1a* expression, in particular for individuals harboring the C allele of the SNP rs6295. This mechanism may lead to impaired serotonin homeostasis. A link between imbalanced serotonergic signaling system and seizure generation has been suggested by multiple studies (Lesch, 2001; Trindade-Filho et al., 2008). Knockout mice lacking 5HT1ARs manifest increased seizure activity, that implicates serotonin receptors in the regulation of neuronal excitability (Sarnyai et al., 2000).

However, susceptibility to seizures may also be increased by multiple other factors such as inflammatory processes. For example, recent studies connect increased neuronal excitability and epileptogenesis to inflammatory processes (Vezzani et al., 2013). Nevertheless, the influence of specific inflammatory factors on seizure frequency is still poorly examined. In order to investigate the correlation of functional parameters dependent of epilepsy including seizures, we analyzed it using the example of inflammatory mediators

known to be implicated in seizure generation and epileptogenesis.

5 TLR4, ATF-3 and IL8 inflammation mediator expression correlates with seizure frequency in human epileptic brain tissue

5.1 Introduction

Recent evidence supports the increased presence and activation of immune and inflammatory pathways as one major factor in epilepsy development (Aarli, 2000; Vezzani and Granata, 2005). Inflammatory responses can be triggered by transient brain damaging events such as neurotrauma, infection, febrile seizures or status epilepticus (Pitkanen and Sutula, 2002; Dinarello, 2004; Bartfai et al., 2007). A contribution to seizure development and maintenance, cell loss and permeability as well as neuronal network hyperexcitability has been claimed in animal models (Friedman et al., 2009; Vezzani et al., 2011; Dedeurwaerdere et al., 2012). Previous immunohistochemical studies suggest a potential release of inflammatory mediators by glia, neurons, endothelial cells of the blood-brain-barrier as well as peripheral immune cells into the brain parenchyma under lesion conditions (Ransohoff et al., 2003; Banks and Erickson, 2010).

Toll-like receptors (TLRs) play a prominent role in the innate immune system in inducing inflammatory processes by enhancing cytokine transcription (Maroso et al., 2010). A major TLR family member, the human Toll-like receptor 4 (TLR4) was first identified in mammalians (Medzhitov et al., 1997) and is mainly expressed in brain (Chakravarty and Herkenham, 2005). In animal studies, TLR4 protein is expressed 1h and 3h after seizure onset in neurons within the pyramidal layer and glial fibrillary acidic protein (GFAP)-positive astrocytes (Maroso et al., 2010). In TLE patients with hippocampal sclerosis TLR4 was found in pyramidal neurons and GFAP-positive astrocytes (Maroso et al., 2010).

TLR-signaling is the main element of innate immune defence. The adaptive response

gene Activating transcription factor 3 (*ATF-3*) represents a key regulator of innate immune responses (Thompson et al., 2009). In recent years, *ATF-3* has received particular attention for being activated by stressful stimuli (Thompson et al., 2009). It interacts with inflammation-related genes, e.g. through down-regulation of *TLR4* or regulation of several cytokines such as interleukin 1 beta, interleukin 6, interleukin 12, tumor necrosis factor alpha and chemokine (C-C motif) ligand 4 (Gilchrist et al., 2006). ATF-3 is upregulated in nuclei of dentate gyrus neurons after injection of the neuroexcitatory stimulant kainate (Francis et al., 2004). In contrast, there is a low ATF-3 expression in intact neurons such as facial motor neurons of mice (Parsadanian et al., 2006).

Cytokines represent key immunological and inflammatory mediators. Interleukin 8 (IL8), also known as CXC Motif Chemokine 8 (CXCL8), is an essential mediator belonging to the Cysteine X Cysteine (CXC) chemokine family (Vlahopoulos et al., 1999). Significantly abundant levels of IL8 are present in patients with severe traumatic brain injuries (Kushi et al., 2003). Another study indicates a correlation of increased levels of IL8 with dysfunction of the blood-brain barrier (Kossmann et al., 1997).

The impact of seizure frequency and additional clinical parameters on gene expression of inflammatory mediators such as *TLR4*, *ATF-3* and *IL8* is largely unknown in human TLE. We here investigate these aspects on the basis of human surgical brain tissue in the subsequent manuscript.

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My contribution to this work includes besides the sample preparation, the main immunohistochemical experiments for assessment of TLR4, ATF-3 and IL8 protein localization in human hippocampal brain tissue and the correlation analyses between gene expression levels of inflammatory mediators and seizure frequency. Additionally, I have

performed the statistical data evaluation and wrote the manuscript.

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Short communication

TLR4, ATF-3 and IL8 inflammation mediator expression correlates with seizure frequency in human epileptic brain tissue

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ABSTRACT

Purpose: Data from animal models has nicely shown that inflammatory processes in the central nervous system (CNS) can modulate seizure frequency. However, a potential relationship between the modulation of seizure frequency and gene expression of key inflammatory factors in human epileptic tissue is still unresolved. Brain tissue from pharmacoresistant patients with mesial temporal lobe epilepsy (mTLE) provides a unique prerequisite for clinico-neuropathological correlations. Here, we have concentrated on gene expression of the human key inflammatory mediators, TLR4, ATF-3 and IL8, in correlation to seizure frequency and additional clinical parameters in human epileptic brain tissue of pharmacoresistant mTLE patients. Furthermore, we characterized the cell types expressing the respective proteins in epileptic hippocampi.

Methods: Total RNAs were isolated from $n = 26$ hippocampi of pharmacoresistant mTLE patients using AllPrep DNA/RNA Mini Kit. cRNA was used for hybridization on Human HT-12 v3 Expression BeadChips with Illumina Direct Hybridization Assay Kit and resulting gene expression data was normalized based on the Illumina BeadStudio software suite by means of quantile normalization with background subtraction. Corresponding human hippocampal sections for immunohistochemistry were probed with antibodies against TLR4, ATF-3, IL8 and glial fibrillary acidic protein (GFAP), neuronal nuclear protein (NeuN) and the microglial marker HLA-DR.

Results: We observed abundant *TLR4* gene expression to relate to seizure frequency per month. For *ATF-3*, we found an inverse correlation of expression to seizure frequency. Lower expression of *IL8* was significantly associated with high seizure frequency. Further, we detected TLR4 expression in neurons and GFAP-positive astrocytes of pharmacoresistant mTLE patients. Only neurons of human epileptic hippocampi express ATF-3. IL8 was expressed in microglia and reactive astrocytes.

Conclusion: Our results suggest a differential correlation of key inflammatory factor expression in epileptic hippocampi and seizure frequency in patients. The modulation of such processes may open new therapeutic perspectives for treating seizures.

1. Introduction

Brain injuries such as neurotrauma, febrile seizures, infections or status epilepticus induce inflammatory responses and this in turn can be associated with acute symptomatic seizures and a high risk of developing chronic epilepsy.[1,2] In human patients only little is known about differential expression of inflammatory factors in correlation to seizure frequency and other clinical parameters. In this context, we have focused on the key human inflammatory mediators, toll-like receptor 4 (*TLR4*), *ATF-3* and *IL8*, for the following reasons: *TLR4* has previously been shown as key trigger of inflammation by inducing the gene transcription of several cytokines and contribute to seizure formation and severity in a TLE animal model.[3] In kainate-induced epileptic mice and in

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hippocampi of pharmacoresistant TLE patients, TLR4 was expressed in pyramidal neurons and GFAP-positive astrocytes.[3] A negative regulator of *TLR4*,[4] the immediate early and adaptive response gene *ATF-3* modulates the expression of genes critically involved in inflammatory responses.[5] ATF-3 expression is dynamically altered by the exposure of cells to many stimuli including epileptic activity in the brain.[6] IL8, as one of the first chemokines identified in the human brain, is known to be expressed exclusively in humans but not in rodents. Previous data suggest expression of IL8 particularly in microglia and astrocytes[7] and an association of intrathecal IL8 synthesis and seizure frequency has been reported.[8] Furthermore, ATF-3 impacts the expression of chemokines.[9] Here, we have used our unique access to human epileptic brain tissue from epilepsy surgery in order to investigate the role of these key mediators of inflammatory processes in correlation to seizure frequency.

2. Materials and methods

We used human bioptic brain tissue to analyze the gene expression data in correlation to the seizure frequency from patients ($n = 26$) with chronic pharmacoresistant mTLE,[10] who underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center. The patient group under study ($n = 26$ Caucasians) is particularly well characterized with respect to seizure documentation and represents substantial differences in seizure frequency (Supplemental Table 1). In all patients, presurgical evaluation using a combination of non-invasive and invasive procedures revealed that seizures originated in the mesial temporal lobe.[11] Surgical resection of the hippocampus was clinically indicated due to pharmacoresistance in every case and all procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University of Bonn Medical Center. Informed written consent was obtained from all patients. Based on screening the Center's fresh frozen hippocampal tissue bank by fresh frozen sections and hematoxylin & eosin staining, care was taken to provide tissue samples representing all hippocampal subfields from the corpus hippocampi for every case included in the present study. Whereas the majority of hippocampi revealed the pattern of Ammon's horn sclerosis (AHS; segmental neuronal cell loss and concomitant astrogliosis and microglia activation), the other hippocampi in the present series lacked the characteristic segmental neuronal cell loss but showed astrogliosis and microglia activation. Those were associated with lesions such as cortical dysplasia or epilepsy-associated tumors. Diagnosis according to international criteria was established by an experienced neuropathologist (AJB) in every case.[12,13] In the course of establishing a nucleic acid bank of respective specimens, genomic DNA and total RNA were isolated from hippocampal tissue samples using AllPrep DNA/RNA Mini Kit (Qiagen, Hilden) according to manufacturer's protocol. For the following analyses, we used only the respective RNA. A total amount of 750 ng cRNA was used for hybridization on Human HT-12 v3 Expression BeadChips with Illumina Direct Hybridization Assay Kit (Illumina, San Diego, CA). The data we extracted for genes of interest was previously analyzed using Illumina's GenomeStudio Gene Expression Module. Gene expression data were normalized using the Illumina BeadStudio software suite by means of quantile normalization with background subtraction. Expression values that were "not detectable" for individual genes, were omitted from the further analysis. Human hippocampal paraffin slices were stained with antibodies against TLR4 (1:20; Santa Cruz Biotechnology, Heidelberg; sc-10741), ATF-3 (1:500; Santa Cruz Biotechnology, Heidelberg; sc-22798), IL8 (1:200; Santa Cruz Biotechnology, Heidelberg; sc-7922) and Glial fibrillary acidic Protein (GFAP) (1:350; Sigma–Aldrich, Munich, Germany; G3893), neuronal nuclear protein (NeuN) (1:500; Millipore, Billerica, MA USA; MAB377) and HLA-DR (1:1000; Dako, Hamburg, Germany; M0746). Correlation analyses between gene expression levels in

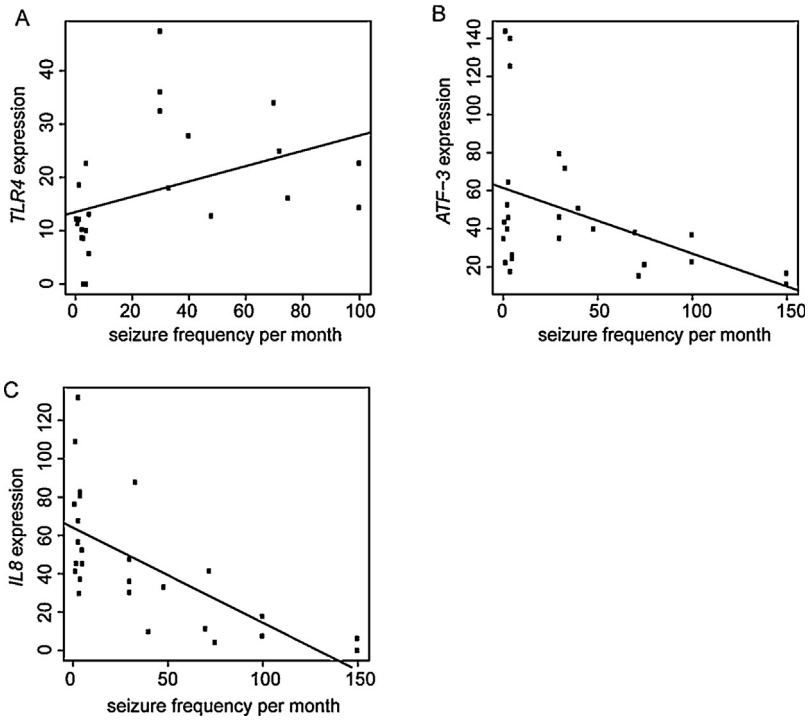

Fig. 1. Relative gene expression of *TLR4*, *ATF-3* and *IL8* in correlation to seizure frequency. (A) Abundant *TLR4* expression significantly correlated with high seizure frequency in patients with pharmacoresistant mTLE ($r = 0.408$; $^{*}p = 0.02$; $n = 24$). (B) Low *ATF-3* expression levels are significantly associated with high seizure frequency in mTLE patients ($r = -0.431$; $^{*}p = 0.01$; $n = 26$). (C) Relative expression data revealed a significant correlation of low *IL8* expression and high seizure frequency ($r = -0.675$; $^{***}p = 1.7 \times 10^{-04}$; $n = 23$). Different n-numbers for the individual genes are due to the fact that expression values that were "not detectable" had to be omitted from the analysis.

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whole hippocampus and variable seizure frequency were done by the Pearson product moment correlation (r) and the correlation t-test (p) using the statistical language R.

3. Results and discussion

We investigated the expression and distribution of the key inflammatory mediators *TLR4*, *ATF-3* and *IL8* in human hippocampal tissue and a potential correlation of seizure frequency and expression of respective genes. We observed that abundant *TLR4* expression correlated significantly with high seizure frequency (Pearson correlation coefficient: $r = 0.407$; two-tailed t-test: $p = 0.047$; $n = 24$; Fig. 1A). For *ATF-3* we found that patients with high seizure frequency show low levels of gene expression ($r = -0.431$; two-tailed t-test: $p = 0.027$; $n = 26$; Fig. 1B). The minor *ATF-3* expression and abundant *TLR4* expression in patients with high seizure frequency may support the role of *ATF-3* as a negative regulator of *TLR4* signaling in human epileptic hippocampi.[4] Furthermore, *ATF-3* has been suggested as a regulator of chemokine expression.[9] Low expression of the human specific IL8 chemokine is significantly correlated with high seizure frequency ($r = -0.671$; two-tailed t-test: $p = 1.7 \times 10^{-04}$; $n = 23$; Fig. 1C). Intriguingly, in our present analyses the human-specific inflammatory mediator IL8 showed the most significant correlation of gene expression and seizure frequency in human epileptic brain tissue. Particularly, these data may underline the need for analyses in human epileptic tissue complementary to animal models, where these analyses for obvious reasons are not possible. In order to address co-correlation of the reported associations of gene expression levels with other clinical parameters, we carried out a comprehensive co-variance analysis of clinical and therapeutic data of the patients included here (Supplemental Table 2). These analyses did not result in significant associations of *TLR4*, *ATF-3* and *IL8* mRNA expression with any of the additional individual parameters investigated. Complementarily, we examined potential inter-correlations among the three inflammatory mediator expression levels based on the seizure frequency. To this end, we performed a one-way MANOVA to test for comprehensive expression dynamics of the inflammatory mediators under study. One-way MANOVA revealed a significant multivariate main effect in the expression of inflammatory mediators based on seizure frequency (Pillai's trace statistic = 2.281, $F(45, 27) = 1.905$, $p = 0.039$). Power to detect the effect was 0.943 (Supplemental Table 3).

Recent studies in hippocampal tissue of pharmacoresistant mTLE patients with hippocampal sclerosis indicated TLR4

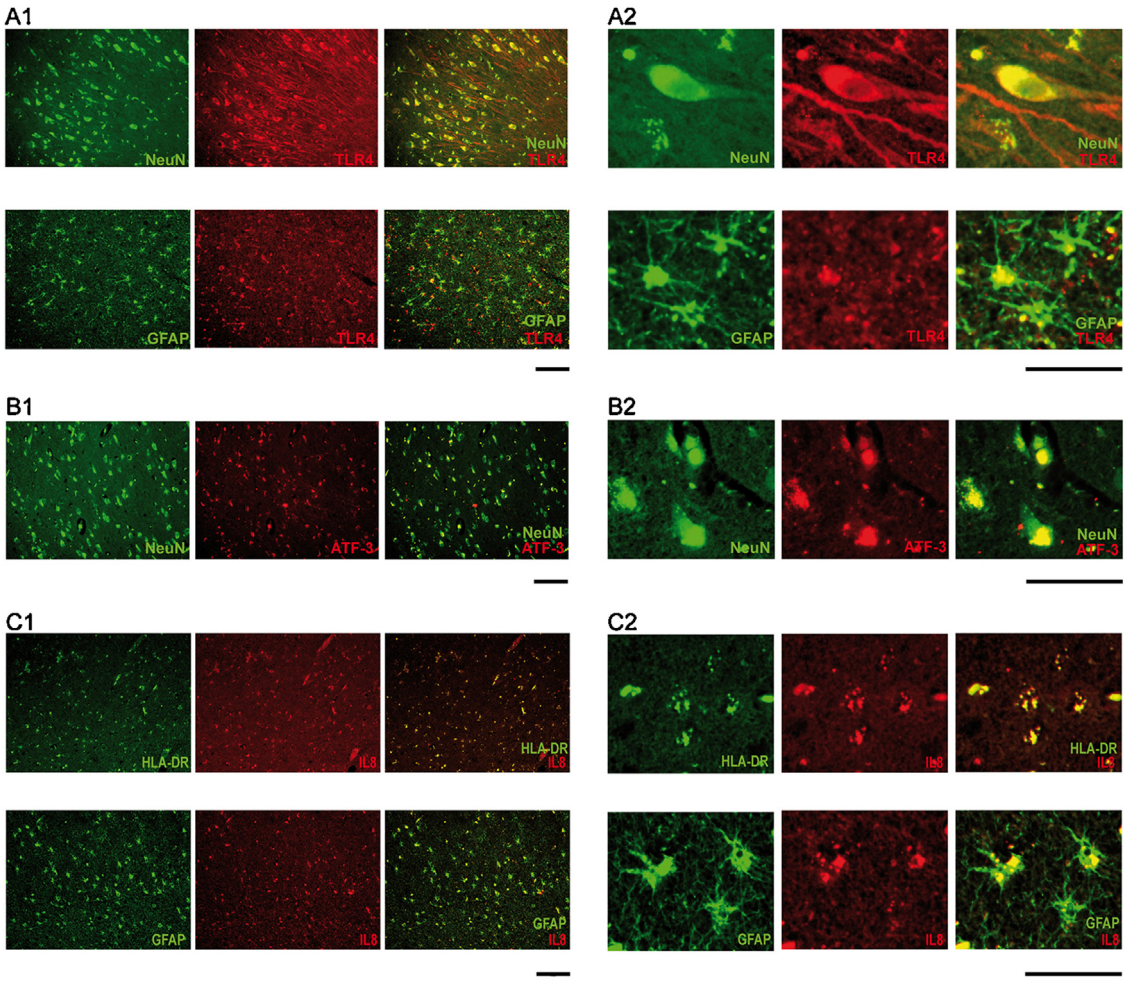

Fig. 2. Immunohistochemical analyses of *TLR4*, *ATF-3* and *IL8* in human hippocampal tissue. (A1) Co-immunoreactions against NeuN and GFAP (both green, as indicated) antibody showed that TLR4 (red) is localized in neurons and reactive astrocytes in mTLE patients (scale bar = 100 μm). (A2) In higher magnification, TLR4 (red signal) is expressed in both, neurons and reactive astrocytes (scale bar = 50 μm). (B1) ATF-3 (red) is localized in neurons (NeuN, green; scale bar = 100 μm) in mTLE patients. (B2) ATF-3 (red) is strongly expressed in representative neurons (scale bar = 50 μm). (C1) Co-immunohistochemistry against the microglial protein HLA-DR (green) and the astrocytic protein GFAP (green) showed that IL8 (red) is expressed in microglia and reactive astrocytes (scale bar = 100 μm). (C2) Higher magnification confirms the presence of IL8 (red) in microglia and reactive astrocytes (scale bar = 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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expression in pyramidal neurons and GFAP-positive astrocytes.[3] We found consistent expression patterns in pharmacoresistant mTLE patients by immunohistochemical reactions with antibodies against GFAP and NeuN (Fig. 2A). We did not observe TLR4 expression in microglia (data not shown). The presence of TLR4 in neurons and reactive astrocytes in human epileptic tissue and the increased expression in patients with high seizure frequency may underline a potential modifying role of TLR4 for seizure frequency in human epilepsy. Previous data suggested expression of ATF-3 in nuclei of dentate gyrus granule cells after kainate induced epilepsy.[14] Glial cells as well as reactive astrocytes lacked ATF-3 expression.[15] By double immunofluorescence, we here observed ATF-3 expression in neurons expressing NeuN in paraffin sections of human hippocampal tissue (NeuN, Fig. 2B). Expression of ATF-3 protein was neither detected in glial components such as reactive astrocytes (data not shown) nor in microglia components (data not shown). In contrast, the chemokine IL8 has been described to be produced exclusively by microglial cells and astrocytes.[7] We performed double-immunofluorescence analyses using antibodies directed against HLA-DR and GFAP to investigate the expression of IL8 in human hippocampal brain tissue. We found IL8 as expressed in both, microglia and reactive astrocytes (Fig. 2C). However, IL8 revealed no co-expression with the neuronal marker NeuN (data not shown).

Our results suggest a substantial correlation between gene expression of inflammatory key mediators and seizure frequency in epileptic patients. Considering the fact that factors under study are differentially expressed in pharmacoresistant mTLE hippocampi, pharmacological interference with such cascades may provide new vistas of anticonvulsive therapy in the future.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

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Pernhorst *et al.*, Supplemental Table 1:

Summary of patient parameters.

(**A**) Parameters including gender, post-operative outcome, pathology and drug therapy are presented in percentage values. The absolute number of available clinical data is indicated in brackets. Age at epilepsy surgery in years, age at seizure onset in years, seizure frequency per month are presented in mean ± SEM values. Information on number of seizures per month relies partially on patients' information and is therefore variable. The post-operative outcome is classified according Engel classification (class I A: completely seizure free; class IV B: no seizure reduction). Biopsy specimens were neuropathologically analyzed according to standard procedures and hippocampi stratified according to the pathological pattern of the patient into the two following groups: AHS (Ammon's horn sclerosis) and lesion associated (ganglioglioma, cavernoma, dysembryoplastic neuroepithelial tumor). Notably, also hippocampi of patients with lesion associated TLE show substantial reactive astrogliosis in the hippocampal formation.

A) Distribution of clinical parameters within sample group.

Number of patients	26
Gender (male vs. female) [26]	57.69% vs. 42.31%
Age at seizure onset in years [22]	15.9 ± 2.5
Seizure frequency per month [26]	39.25 ± 9.74
Drug therapy (Sodium-channel blockers monotherapy vs. LEV-combinations vs. non LEV-combinations) [25]	28% vs. 36% vs. 36%
Age at epilepsy surgery in years [26]	33.73 ± 3.42
Post-operative outcome (Engel IA vs. Engel IV B) [24]	62.5% vs. 37.5%
Pathology (AHS vs. lesion associated) [26]	57.69% vs. 42.31%

Pernhorst *et al.*, Supplemental Table 2

The gene expression of the inflammatory key mediators *TLR4*, *ATF-3* and *IL8* in correlation to clinical parameters.

The co-variance analyses of expression of *TLR4, ATF-3* and *IL8* did not result in significant correlation with individual clinical parameters. Variables are presented in mean ± SEM values.

A) Clinical data of TLE patients: correlation to gene expression of *TLR4*

Clinical variables	Mean	SEM	P
Gender (Male / Female)	17.65 / 16	2.41 / 3.94	0.72
Age at seizure onset in years (1-10 / 11-50)	18.69 / 15.97	4.1 / 3.42	0.639
Age at epilepsy surgery in years (1-10 / 11 and more)	11.22 / 17.31	0.75 / 2.62	0.41
Seizure-free post-operative (I A / IV B)	15.03 / 19.09	3.28 / 2.78	0.422
Pathology (AHS / lesion associated)	18.9 / 14.29	3.08 / 2.79	0.315

B) Clinical data of TLE patients: correlation to gene expression of *ATF-3*

Clinical variables	Mean	SEM	P
Gender (Male / Female)	56.97 / 37.29	11.35 / 4.21	0.181
Age at seizure onset in years (1-10 / 11-50)	34.49 / 57.84	5.81 / 9.95	0.116
Age at epilepsy surgery in years (1-10 / 11 and more)	22.48 / 49.43	7.22 / 6.89	0.175
Seizure-free post-operative (I A / IV B)	40.07 / 56.05	3.95 / 15.05	0.242
Pathology (AHS / lesion associated)	47.15 / 50.67	7.46 / 13.18	0.858

C) Clinical data of TLE patients: correlation to gene expression of *IL8*

Clinical variables	Mean	SEM	P
Gender (Male / Female)	48.19 / 40.25	8.74 / 7.03	0.589
Age at seizure onset in years (1-10 / 11-50)	37.51 / 56	14.59 / 5.66	0.219
Age at epilepsy surgery in years (1-10 / 11 and more)	13.13 / 47.61	8.28 / 6.8	0.084
Seizure-free post-operative (I A / IV B)	41.02 / 45.99	6.55 / 11.76	0.717
Pathology (AHS / lesion associated)	50.45 / 36.88	7.93 / 10.56	0.338

D) Correlation of the *TLR4* gene expression to antiepileptic drug treatment using Bonferroni's Multiple Comparison Test.

	Mean difference	t	P value
Sodium-channel blockers mono-therapy vs. LEV-combinations	1.931	0.2823	P > 0.05
sodium-channel blockers mono-therapy vs. non-LEV combina-tions	5.968	0.8516	P > 0.05
LEV-combinations vs. non-LEV combinations	4.037	0.6403	P > 0.05

E) Correlation of the *ATF-3* gene expression to antiepileptic drug treatment using Bonferroni's Multiple Comparison Test.

	Mean difference	t	P value
Sodium-channel blockers mono-therapy vs. LEV-combinations	-13.13	0.8031	P > 0.05
Sodium-channel blockers mono-therapy vs. non-LEV combina-tions	17.57	1.049	P > 0.05
LEV-combinations vs. non-LEV combinations	30.70	2.037	P > 0.05

F) Correlation of the *IL8* gene expression to antiepileptic drug treatment using Bonferroni's Multiple Comparison Test.

	Mean difference	t	P value
Sodium-channel blockers mono-therapy vs. LEV-combinations	-1.387	0.07012	P > 0.05
Sodium-channel blockers mono-therapy vs. non-LEV combina-tions	-4.431	0.2692	P > 0.05
LEV-combinations vs. non-LEV combinations	-3.044	0.1602	P > 0.05

Pernhorst *et al.*, Supplemental Table 3

One-way MANOVA statistics comparing expression level of inflammatory mediators and seizure frequency. Parameters including the value of the Pillai's trace test (Value), the overall *F* statistic over all three dependent expression variables (*F*), the number of degrees of freedom in the model (Hypothesis df), the number of degrees of freedom associated with the model errors (Error df), the significance of the F statistic given as P value (Significance P value) and the statistical power to detect the effect (Observed Power).

Effect	Statistic	Value	*F*	Hypothesis df	Error df	Significance P value	Observed Power
Seizure frequency	Pillai's Trace	2.281	1.905	45	27	0.039	0.943

5.3 Summary

In our study, we investigated the role of inflammatory mediator gene expression levels in the context of seizure propensity and other clinical aspects of epilepsy. We observed a differential correlation of mRNA expression levels of inflammatory mediators including *TLR4*, *ATF-3* and *IL8* and seizure frequency in human surgical brain tissue of TLE patients. In order to correlate gene expression levels and seizure frequency of TLE patients, we stratified between low and high seizure frequency. Here, we defined low seizure frequency as a range of 0 to 10 seizures per month, whereas high seizure frequency is given by 30 to more than 100 seizures per month.

Our study reveals a significant impact of seizure frequency on *TLR4* expression. *TLR4* expression is significantly increased in a group of TLE patients characterized by high seizure frequency compared to patients with low seizure frequency. Our data indicates TLR4 protein expression in neuronal as well as astrocytic components of hippocampal brain tissue, confirming previous data (Maroso et al., 2010). Interestingly, the increased *TLR4* expression in patients with high seizure frequency may suggest this molecule as target for modulating seizure frequency in human epilepsy. As shown by Maroso *et al.*, an epileptogenic insult may stimulate *TLR4* activation which is then sustained by the recurrence of epileptic activity (Maroso et al., 2010).

Interestingly, *ATF-3* as a negative regulator of *TLR4* shows a significantly less abundant gene expression in patients with high seizure frequency. In immunohistochemical labelings, we detected ATF-3 protein expression in neurons of human epileptic hippocampi but neither in glial nor microglia components in accordance with a recent report (Ohba et al., 2003). *ATF-3* is an immune regulator that functions in a context-dependent manner, particularly by negative modulation of the TLR-signaling pathway (Gilchrist et al., 2006) or by positive regulation of various cytokine and chemokines of the interleukin family, Tumor necrosis factor alpha (TNFα) and Chemokine (C-C motif) ligand 2 (CCL2) (Zmuda et al., 2010).

A rodent homologue of human chemokine *IL8* has not been identified to date and so it is denoted as human-specific. Intriguingly, *IL8* displays the strongest correlation of gene expression and seizure frequency. We found that low expression of the human specific *IL8* is significantly correlated with high seizure frequency. This result may demonstrate the relevance of analyzing molecular pathomechanisms in human surgical brain tissue complementary to animal models. Immunohistochemical labelings with antibodies directed against the microglia marker HLA-DR as well as the astrocytic marker GFAP indicate both IL8 protein expression in microglia components and in reactive astrocytes. The release of IL8 from microglia and reactive astrocytes in response to infection or injury is established (Liu and Hong, 2003; Farina et al., 2007).

In conclusion, we demonstrate a significant correlation of expression of distinct mediator genes of inflammation to seizure frequency in human epileptic brain tissue. With respect to differential expression of inflammatory factors in human hippocampal tissue of TLE patients, our results highlight the possibility to interfere with inflammatory signaling pathways as potential new targets for therapeutic intervention, particularly for epileptic patients not responding to antiepileptic drugs.

6 Discussion

Epileptic seizures occur in a variety of different disorders. In *generalized* epilepsies 'mutational channelopathies' have been frequently described (Weiergräber et al., 2008; Nabbout and Scheffer, 2013). These forms of epileptic disorders are generally rare. In more frequent *focal* epilepsies, changes on mRNA level of genes including ion channels ('transcriptional channelopathies') and neurotransmitter receptors have been described, whereas mutations of respective genes are rare (Oliveira et al., 2011). Focal epilepsies represent multifactorial disorders. So, pathogenetic factors, episodically shifting the brain over a virtual threshold to the emergence of seizures, are *individually* neither necessary nor sufficient.

In recent years, several SNPs located in putative promoter regions have been claimed as accumulated in episodic CNS disorders. Transiently altered expression of corresponding genes therefore constitutes a potential pathogenetic aspect for the manifestation of episodic symptoms. The availability of biopsy tissue from epilepsy surgery of pharmacoresistant TLE patients provides a unique prerequisite in order to study the potential impact of gene promoter variants on transcription as well as the abundance of molecules involved in neurotransmission and immune responses in concert with stratification of patients according to clinical parameters. Based on these considerations, we have explored several key pathogenetic aspects of epilepsy in respective human tissue samples.

On the molecular level, aberrant amounts of both excitatory, e.g. glutamate, and inhibitory, e.g. GABA, neurotransmitters have been observed in epilepsies. McNamara *et al.* suggested that seizures are caused by a fundamental imbalance between excitation and inhibition (McNamara, 1994).

Increasing evidence also suggests a role of serotonin in seizure development (Bagdy et al., 2007). Altered concentrations of 5-HT receptors may directly or indirectly result in neuronal dysfunction (Barnes and Sharp, 1999). Likewise, Cavalheiro *et al.* indicate an increased synthesis and utilization rate of hippocampal serotonin during a

first SE (Cavalheiro et al., 1994). In humans with complex partial seizures, exposure of fluoxetine, a serotonin reuptake inhibitor, leads to a decrease in seizure frequency (Favale et al., 1995).

In order to study the contribution of genetic variability to transcriptional dynamics in epilepsy, we examined allelic promoter variants in human biopsy brain tissue of pharmacoresistant TLE patients. Due to the complexity of neuropathological changes in TLE hippocampi, the involvement of multiple genes and signaling cascades in the pathogenesis of TLE appears obvious. Numerous associations of genetic variations, in particular SNPs, have been linked to brain dysfunction in general before (Urak et al., 2006; Lorenz et al., 2006; Marziniak et al., 2007). Since such alterations have been prominently shown in genes related to GABA-homeostasis and serotonergic transmission, we have concentrated in two studies of this thesis on respective pathways.

6.1 Distribution of allelic variants in distinct patient cohorts

Case-control studies represent one of the most common study designs in order to test for disease associated allelic variants. Divergent allelic or genotypic distribution between either diseased individuals or healthy controls highlights potential risk variants. Here, we initially genotyped DNA of pharmacoresistant patients suffering from TLE to detect respective SNPs associated with brain disorders such as epilepsy, depression and migraine, the latter also being potential comorbidities of focal epilepsies.

Our data revealed a significantly abundant C allele in the *ALDH5A1* promoter SNP rs1883415 when comparing the TLE-case group to a control group. Given the substantial prevalence rate of neuropsychiatric disorders concomitting epilepsies, particularly in case of major depressive episodes accompanying TLE (Swinkels et al., 2005; Kondziella et al., 2007; Kanner, 2008), we further analyzed in the TLE patient cohort the existence of depressive symptoms. In default of a large number of brain samples of TLE patients with depressive symptoms, we extended the TLE patient population under study with

blood DNA samples from additional TLE patients with depression. The frequency of the SNP rs1883415 C allele in TLE patients with depression is significantly increased compared to TLE patients lacking depressive symptoms.

In contrast, for *GABRB3* promoter A/G variation rs4906902, we observe no significant association for both alleles comparing TLE patient group and controls. Previously reported associations indicated that the G allele as well as the A allele of SNP rs4906902 are differentially represented in patients with neurological disorders. For example, the G allele is overrepresented in CAE (Urak et al., 2006), but could not be found in remitting CAE patients when compared with healthy controls (Tanaka et al., 2012a). With respect to the potential comorbidity of neuropsychiatric disorders to epilepsy, we demonstrated that the G allele is more frequent in TLE patients with depression as compared to patients showing no depressive symptoms. However, Feusner *et al.* reported an association of the A allele to depression in patients with post-traumatic stress disorder (Feusner et al., 2001). Given that depressive symptoms occur in different pathologic contexts, alleles of *GABRB3* promoter SNP rs4906902 may be differentially overrepresented in depressive patients in a context dependent manner.

As previously shown, SNP rs6295 is associated with mood disorders including major depression and bipolar disorder (Kishi et al., 2009, 2011). In fact, we neither detected significant associations of particular SNP rs6295 allele nor genotype in TLE patients versus non-epileptic controls (Schmermund et al., 2002). Likewise, we determined no significant SNP rs6295 genotype as well as allele assocation in TLE patients with depression compared to TLE patients without depression.

The allelic variants in the *HTR1A* promoter did in contrast not correlate to clinical parameters in TLE patients. In a following step, we correlated the presence of promoter polymorphisms to gene expression.

6.2 Impact of promoter SNPs on gene expression in episodic brain diseases

About 30% of TLE patients are pharmacoresistant. Those TLE patients usually undergo surgical therapy for effective seizure control (Engel, 1996b; Regesta and Tanganelli, 1999).

Direct analysis of expression levels in diseased human surgical brain tissue specimens provides a useful means to study allele-specific alterations in episodic brain diseases. Thereby, we found that *ALDH5A1* gene expression in TLE patients homozygous for the A allele in the SNP rs1883415 to be significantly less abundant compared to TLE patients homozygous for the C allele. Allele-specific expression of *ALDH5A1* in TLE patients stratified for the SNP rs1883415 genotype may relate to differential expression of the enzyme SSADH. Reduced expression of SSADH and therefore lower turnover of GABA, is at least partially comparable to SSADH deficiency which is associated with clinical features such as generalized tonic-clonic and atypical absence seizures (Pearl et al., 2003). With respect to an established relationship of disturbed GABA homeostasis with epileptogenic effects (Wong et al., 2003), our results reflect an interesting aspect in neurotransmission of epileptic brain.

For *GABRB3* and the SNP rs4906902, TLE patients with the GG genotype exhibit a significantly decreased *GABRB3* mRNA expression compared to TLE patients with the AA genotype. This allele-dependent regulation of *GABRB3* expression may reflect an immediate effect on neurotransmission. As previously reported, reduced levels of *GABRB3* are accompanied by decreased levels of inhibitory $GABA_A$ receptors and therefore may relate to an increase of seizure susceptibility (Tanaka et al., 2012b). With respect to allele-specific expression differences of *ALDH5A1* and *GABRB3*, it has to be considered, that such differences may only manifest in situations of challenged neuronal network conditions. Episodes such as febrile states or encephalitis *per se* represent potentially hyperexcitable states with particular critical needs for inhibitory

transmitter systems. Impaired turnover and also only slightly altered signaling of a main inhibitory neurotransmitter system may under such conditions manifest as failure of network inhibition and contribute to the emergence of epileptic seizures. In this context, our present observations may well contribute as explanation of the episodic nature of epileptic symptoms.

Alterations in the serotonergic pathway can also contribute to the emergence of epilepsy. Didelot *et al.* reported impaired 5-HT receptor binding in mesial temporal structures of patients suffering from TLE (Didelot et al., 2008). As shown in section 4, we detected allele-specific expression of *HTR1a*, a gene that encodes for a G protein-coupled serotonin receptor. In the present study, individuals with the SNP rs6295 CC genotype have more abundant *HTR1a* mRNA expression compared to patients homozygous for the SNP rs6295 G allele. Irregular *HTR1a* expression levels lead to dysregulation of 5-HT release and therefore to an impaired serotonin homeostasis. Numerous studies also suggest that disturbances in the serotonergic system are related to neuropsychiatric disorders including epilepsy, aggression, depression, anxiety and schizophrenia (Savic et al., 2004; Siever, 2008; Filip and Bader, 2009). However the isolated notion of a correlation of allelic variants in gene promoters and corresponding gene expression in human brain tissue would be rather incomplete. Expressed transcription factors potentially binding at promoter motifs should be taken into consideration. This step of complexity we also aimed at in the studies of this thesis. These experiments had implications as follows.

6.3 Allele-specific transcriptional regulation

Prior research emphasizes the impact of SNPs found in regulatory gene regions on transcriptional modulation by either gene splicing, messenger RNA degradation, changes in transcription factor binding or promoter activity (Lohrer and Tangen, 2000). Therefore, we examined potential binding sites of transcription factors in given proximity of

respective SNP positions. Subsequently, we investigated TF binding affinity in dependence on the SNP. Bioinformatic predictions of potential TFBSs using position-specific scoring matrices suggest differential allele-dependent binding affinity of regulatory TFs. Concerning the SNP rs1883415 in the *ALDH5A1* promoter, we identified a potential TFBS for Egr-3. Egr-3 revealed significantly higher binding affinity to the C allele as compared to the A allele. This result coincides with the substantially stronger C allele promoter activation after exposure of the TF Egr-3 using luciferase reporter assays in section 3. Honkaniemi *et al.* examined an increased *Egr-3* mRNA expression level in a rat TLE model after exposure to kainic acid to induce status epilepticus (Honkaniemi and Sharp, 1999). Our data would be in line with an enhanced stimulation by Egr-3 on *ALDH5A1* expression level in patients particular homozygous for the C allele. Experimental evidence also suggests that Egr-3 regulates expression of the α4 subunit of the $GABA_A$ receptor in primary hippocampal neurons and in TLE models (Brooks-Kayal et al., 1998; Roberts et al., 2005). Certain TFs may, therefore, orchestrate several neurotransmitter systems in epileptogenesis in a highly complex fashion and manifest overadditive effects.

Furthermore, stimulation of *GABRB3* activity by the TF MEF-2 demonstrated significantly increased promoter activity for the A allele in contrast to the G allele promoter. This is in line with our previous prediction of a MEF-2 binding motif at the SNP rs4906902. The predicted MEF-2 motif exhibits a more relevant sequence complementarity to the A allele promoter variant in comparison to the G allele variant. MEF-2 is highly expressed in brain. MEF-2 can be induced by extracellular stimuli, leading to activation of a gene expression program to modulate an excitatory synapse activation on hippocampal neurons (Flavell et al., 2006). Additionally, MEF-2 was shown as reduced in hippocampal tissue of pilocarpine-treated SE-rats at the chronic stage compared to control rats (own unpublished data). In the light of these data, an attenuated activation of *GABRB3* promoter may be the consequence in TLE.

With regard to the *HTR1a* promoter, we found that the SNP rs6295 is present in a

potential binding region of transcriptional repressors as well as an activator. Former studies reveal repressing effects on *HTR1a* by TFs NUDR and Hes5 (Lemonde et al., 2003; Albert et al., 2011). Intriguingly, our data demonstrates a potential TFBS for activating TF c-Jun. c-Jun has a higher binding affinity to the SNP rs6295 C variant compared to G variant in the *HTR1a* promoter. Furthermore, this result is supported both by c-Jun *in vivo* binding to *HTR1a* promoter as shown by ChIP experiments as well as stronger activation of the C allele promoter by c-Jun using luciferase reporter assays. Considering the interaction of activating and inhibitory TFs in eukaryotic gene transcription, we preferentially examined effects of the transcriptional repressor Hes5 using our own bioinformatic TFBS prediction and *in vitro* luciferase reporter assays. Both transcriptional regulators Hes5 and c-Jun have interfering effects on the *HTR1a* promoter. c-Jun reverses the repression of Hes5 in both *HTR1a* promoter variants. In particular in the *HTR1a* C variant this effect is substantially stronger as compared to the *HTR1a* G variant.

Experimental evidence points toward an upregulation of c-Jun at early timepoints in the lithium-pilocarpine model to induce SE in mature brain of rats, which returned to basal level 24 hours after treatment (Han et al., 2009). Either increased stimulating efficacy of c-Jun or reduced inhibitory effects of Hes5 on the C variant may play a role here. With respect to investigations of respective effects of those repressors and activators in migraine and depression, our approach reaches certain limitations. Concerning the lack of availability of respective brain tissue samples, it generally needs to be considered that the expression of particularly repressing and activating transcription factors may be dynamic in time dimensions and also in different anatomical brain regions. Respective changes may, however, be well extrapolated from animal models and be analyzed complementary to data obtained from human biopsy brain tissue. The expression levels and dynamics of repressing and activating TFs on allelic SNP variants in gene promoters with different binding affinities contribute a high level of complexity to gene promoter control in brain tissue of individual humans. In contrast to the dynamic

transcriptional changes in brain tissue, genetic variability provides a rather stable trait of individuals to be correlated to clinical aspects of patients. These aspects may be considered when correlating allelic variants and gene expression to clinical parameters.

6.4 Clinico-genetic correlations

Our data show allele-specific mRNA expression of genes involved in GABA-ergic or serotoninergic homeostasis. It can be derived from multiple studies that external factors may influence gene expression in epilepsy. As demonstrated by Wang *et al.* or by Christensen *et al.*, antiepileptic drugs such as lamotrigine or levetiracetame have effects on respective gene expression (Wang et al., 2002; Christensen et al., 2010). Other clinical parameters such as pathology (Teocchi et al., 2013) or age (Liao et al., 2010) are also associated with potential gene expression changes.

With respect to previously studied associations of gene expression and distinct SNP genotypes with comorbidities and clinical parameters, we analyzed clinical data for gender, age at seizure onset, number of seizures per month, age at epileptic surgery, pathological pattern, post operative outcome according to Engel classification, incidence of depression disorder and antiepileptic and antidepressant drug therapy for TLE patients in our collective (section 3.1 Supplemental Table 2, section 4.1 Supplemental Table 1 and section 5.1 Supplemental Table 1).

Considering that the evaluation of clinical data partially relies on subjective patient information, patient samples under study are somewhat heterogeneously characterized. We performed comprehensive covariance analysis of clinical, neuropathological patterns and pharmacotherapy data of our patients.

Neither therapeutic variables including antiepileptic and antidepressant drugs nor clinical parameters such as age at onset of epilepsy, gender and age at epilepsy surgery reveal significant effects on gene expression of both *ALDH5A1* and *GABRB3* (section 3.1 Supplemental Table 4 and 5). The same holds true in covariance analyses between

HTR1a expression and clinical parameters. None of the investigated variables indicate a significant impact on *HTR1a* expression as shown in section 4 Supplemental Table 3 and 4. Our data also showed no significant association of any particular SNP genotypes with the individual parameters investigated.

Our data do not argue in favor of an effect of clinical and therapeutical variables on gene expression nor SNP genotypes in human surgical brain tissue of TLE patients. The presented evidence thus emphasizes the impact of the SNP rs1883415, rs4906902 as well as rs6295 to affect corresponding gene expression in human surgical brain tissue of TLE patients independent of covariance of clinical variables under study.

6.5 Correlation of molecular pathological parameters to seizure frequency

The most compromizing clinical feature of chronic epilepsies are certainly seizure attacks in affected patients. It represents an important but also very challenging aspect to obtain reliable data on seizure frequency in epileptic patients obviously not being permanently monitored in a clinical context. Nevertheless, data obtained on seizure frequency over a time course of e.g. several months before epilepsy surgery represents a highly valuable parameter to be correlated to pathological aspects in surgical brain tissue samples.

In this context, multiple studies confirmed the link between seizures and inflammatory molecules (Vezzani et al., 2000; Maroso et al., 2011). Microglial and astrocytic cells are activated by seizures to modify inflammatory processes such as upregulation of genes associated with immune and inflammatory responses including chemokines and cytokines (Aronica and Gorter, 2007; Wetherington et al., 2008; Ravizza et al., 2008). Here, we observed a significant correlation of gene expression of inflammatory mediators and seizure frequency in human surgical brain tissue of TLE patients. Abundant *TLR4* expression significantly correlates with high seizure frequency. The potential

role of *TLR4* as regulator in episodic brain disorders including epilepsy is supported by TLR4 protein expression in neurons and astrocytes in epileptic tissue as well as the significant correlation of *TLR4* mRNA expression and seizure activity. This finding in human brain tissue is in a good agreement to data from animal models showing an epileptogenic affect of TLR4 (Maroso et al., 2010). Our present data may nevertheless add an important aspect to the existing knowledge on TLR4 in the context of TLE, i.e. the substantially increased expression in brain tissue that still shows a particular high propensity for the generation of epileptic seizures even at very advanced stages of the disease. Respective data may be rather valuable in order to translate potential antiepileptic therapies targeted to TLR4 into the context of chronic human focal epilepsies.

In contrast to *TLR4*, *ATF-3*, inducible by stress-stimuli such as seizures (Chen et al., 1996; Thompson et al., 2009), is expressed at only low level in patients suffering from high seizure frequency. In accordance to the role of ATF-3 as neuronal marker of nerve injury in the nervous system (Tsujino et al., 2000), we present ATF-3 accumulation in neuronal components of human epileptic hippocampal tissue. Considering seizure frequency, *TLR4* and *ATF-3* are reciprocally expressed and these dynamics may be in line with a role of *ATF-3* as negative regulator of *TLR4* (Gilchrist et al., 2006; Suganami et al., 2009).

Interestingly, the human-specific chemokine *IL8* in our respective study exhibits strongest correlation of seizure frequency and mRNA expression. We found IL8 protein expression in microglia and reactive astrocytes as also demonstrated in studies of Ehrlich *et al.* and Hesselgesser *et al.* in epileptic tissue derived from pharmacoresistant TLE patients (Ehrlich et al., 1998; Hesselgesser and Horuk, 1999). Microglial cells and also astrocytes are present at early stages after epileptogenic insults as well as still activated at chronic stages of focal epilepsies. The fact that with IL-8 a molecule shows a strong correlation of expression and seizure frequency, underlines the relevance of respective study approaches to human epileptic tissue. The access to such tissue samples allows

the characterization of molecules, that exhibit a particular abundance in epileptic foci with seizure activity. The fact that IL-8 is a human specific gene makes it obviously impossible to carry out analyses of this important inflammatory factor in rodent models. However, studies in human tissue samples are certainly restricted to chronic stages of the disease. Whether IL-8 is also a potential antiepileptogenic target can not be accessed in human tissue samples. Therefore the development of humanized transgenic mice may be considered in the future.

Another aspect of inflammation in epileptic tissue relates to mechanisms by which hyperexcitability is mediated. Recent data point to a role for modulation of neurotransmission. Hu *et al.* report on contribution of inflammation to hyperexcitability by inhibiting astrocytic glutamate reuptake (Hu et al., 2000). Viviani *et al.* show inflammatory mediators reducing GABA-mediated chloride currents or $GABA_A$ receptor expression at neuronal membranes in order to anticipate reduction of GABA-mediated inhibition in inflamed brain tissue (Viviani et al., 2007).

The publications, on which the present thesis is based, provide novel insights in the relevance of dynamic expression of inflammatory factors and neurotransmission-related molecules in epileptic tissue.

The potential of extrapolating such approaches on gene expression and corresponding promoter regulation in a certain disease context onto a genome wide scale has become available only recently.

7 Outlook

Considering the high complexity of the brain and neurological disorders including epilepsy, depression and migraine, comprehensive molecular genetic approaches may be excellently suited to understand better multilayered gene regulation processes in human diseased brain tissue. Within the framework of this thesis we have generated

data on SNPs, transcript abundance and promoter methylation on a genome wide level from human biopsy epileptic tissue, which now forms the basis for further comprehensive analyses.

Identification of expression quantitative trait loci (eQTL) as well as methylation quantitative trait loci (mQTL) will provide intriguing insights in the relevance of both *cis* and *trans* effects of SNPs and methylation on gene expression in the human brain. Ultimately, resulting data sets will foster the identification of gene networks involved in multifactorial brain disease pathogenesis.

8 Publications

8.1 Research articles

Pernhorst K, Raabe A, Niehusmann P, van Loo KM, Grote A, Hoffmann P, Cichon S, Sander T, Schoch S, Becker AJ. Promoter variants determine -aminobutyric acid homeostasis-related gene transcription in human epileptic hippocampi. J Neuropathol Exp Neurol. 2011 Dec; 70(12):1080-1088. PubMed PMID: 22082659.

Pernhorst K, van Loo KM, von Lehe M, Priebe L, Cichon S, Herms S, Hoffmann P, Helmstaedter C, Sander T, Schoch S, Becker AJ. Rs6295 promoter variants of the serotonin type 1A receptor are differentially activated by c-Jun *in vitro* and correlate to transcript levels in human epileptic brain tissue. Brain Res. 2013 Mar 7; 1499:136-144. Epub 2013 Jan 16. PubMed PMID: 23333373.

Pernhorst K, Herms S, Hoffmann P, Cichon S, Schulz H, Sander T, Schoch S, Becker AJ, Grote A. TLR4, ATF-3 and IL8 inflammation mediator expression correlates with seizure frequency in human epileptic brain tissue. Seizure. 2013 May 22. doi:pii: S1059-1311(13)00144-1. 10.1016/j.seizure.2013.04.023. [Epub ahead of print] PubMed PMID: 23706953.

8.2 Poster presentations

Pernhorst K, van Loo K, Grote A, Schoch S, Becker AJ. Allelic promoter variants accumulated in epileptic patients: indication of mRNA-expression alterations of corresponding genes. Symposium I Genomics of Common Disease P-1-8. 2nd Annual Meeting of NGFN-Plus and NGFN-Transfer

Pernhorst K, van Loo K, Raabe A, Niehusman P, Grote A, Schoch S, Becker AJ.

Compromized expression control of genes involved in serotonin and GABA metabolism in human epileptic tissue. Symposium I Genomics of Common Disease P-I/II-066. 3rd Annual Meeting of NGFN-Plus and NGFN-Transfer

Pernhorst K, Raabe A, Niehusman P, van Loo KMJ, Grote A, Hoffmann P, Cichon S, Sander T, Schoch S, Becker AJ. Promoter variants determine GABA-related transcription in human epileptic brain. Symposium I Genomics of CNS Disorders P-I-22, 4th Annual Meeting of NGFN-Plus and NGFN-Transfer

Pernhorst K, Raabe A, Niehusman P, van Loo KMJ, Grote A, Hoffmann P, Cichon S, Sander T ,Schoch S, Becker AJ. Aberrant transcription of serotonin- and GABA-homeostasis related genes due to accumulated promoter variants in human episodic brain disorders. Epigenomics of Common Diseases P 59

Pernhorst K, van Loo KM, von Lehe M, Priebe L, Cichon S, Herms S, Hoffmann P, Helmstaedter C, Sander S, Schoch S, Becker AJ. Rs6295 promoter variants of the serotonin type 1A receptor correlate to respective mRNA expression in human epileptic brain tissue. Symposium I Genomics of Common Disease P-III-14, 5th Annual Meeting of NGFN-Plus and NGFN-Transfer

9 List of figures

10 List of abbreviations

5HT1AR 5-HT1A receptor

5-HT 5-hydroxytryptamine

AED Antiepileptic drug

AHS Ammon's horn (or hippocampal) sclerosis

ALDH5A1 Aldehyde dehydrogenase 5 family, member A1

ATF-3 Activating transcription factor 3

BBB Blood-brain barrier

bp Base pair

CAE Childhood absence epilepsy

CCL2 Chemokine (C-C motif) ligand 2

ChIP Chromatin immunoprecipitation assay

ChIP-Seq Chromatin Immunoprecipitation Sequencing

CNS Central nervous system

CNV Copy number variant

CpG Cytosine phoshpodiester bond Guanine

CXC Cysteine X Cysteine

CXCL8 CXC Motif Chemokine 8

DNA Deoxyribonucleic acid

DPE Downstream promoter element

EBI European Bioinformatics Institute

EEG Electroencephalogram

Egr-3 Early growth response 3

EMBL European Molecular Biology Laboratory

EPD Eukaryotic Promoter Database

eQTL Expression quantitative trait loci

GABA Gamma-aminobutyric acid

GABRA5 Gamma-aminobutyric acid A receptor, alpha 5

GABRB3 Gamma-aminobutyric acid A receptor, beta 3

GABRG3 Gamma-aminobutyric acid A receptor, gamma 3

GFAP Glial fibrillary acidic protein

GHB Gamma-hydroxybutyric acid

GWA Genome-wide association

HTR1a 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled

IGE Idiopathic generalized epilepsy

IL8 Interleukin 8

INR Initiator sequence

JME Juvenile myoclonic epilepsy

kb kilo base

MEF-2 Myocyte enhancer factor 2

MPromDb Mammalian Promoter Database

mQTL Methylation quantitative trait loci

mRNA Messenger ribonucleic acid

NCBI National Center for Biotechnology Information

PFM Position frequency matrix

PSSM Position-specific scoring matrix

PWM Position weight matrix

rs RefSNP

RT-PCR Reverse transcription polymerase chain reaction

SE Status epilepticus

SNP Single nucleotide polymorphism

SSADH Succinic semialdehyde dehydrogenase

TFBS Transcription factor binding site

TFIIA Transcription factor IIA

TFIIB Transcription factor IIB

TFIID Transcription factor IID

TFIIE Transcription factor IIE

TFIIF Transcription factor IIF

TFIIH Transcription factor IIH

TF Transcription factor

TLE Temporal lobe epilepsy

TLR Toll-like receptor

TLR4 Toll-like receptor 4

TNFα Tumor necrosis factor alpha

TNR Trinucleotide repeat

TRANSFAC Transcription Factor database

TSS Transcription start site

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